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TAKAHIRO KANAGAWA

1990

**DEVELOPMENT OF MICROBIAL DEODORIZING
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ABBREVIATIONS

MM : methyl mercaptan
DMS : dimethyl sulfide
DMDS : dimethyl disulfide
DMDTP: O,O-dimethyl phosphorodithioate
DMTP : O,O-dimethyl phosphorothioate
DEDTP: O,O-diethyl phosphorodithioate
DMP : dimethyl phosphate
DEP : diethyl phosphate
MP : monomethyl phosphate
EP : monoethyl phosphate
P_i : inorganic orthophosphate
TOC : total organic carbon
MLSS : mixed liquor suspended solids
A₆₆₀ : absorbance at 660 nm
TLC : thin-layer chromatography

INTRODUCTION

Methyl mercaptan (MM), dimethyl sulfide (DMS), dimethyl disulfide (DMDS), and H_2S are malodorous compounds which exceed the odor threshold at low concentrations (1). They are produced by the wood pulping industry, in oil refineries, and in manure and sewer systems and cause malodorous air pollution. Various methods have been used for the removal of malodorous compounds from contaminated air. The biological deodorizing method is now attracting attention because of its low operation cost. However, its removal efficiency for sulfur-containing malodorous compounds is reported to be poor (2,3). There is little knowledge available on the microorganisms which cause the degradation of methyl sulfides. MM was degraded with a biofilter made of pine bark (4), but there is no direct evidence of any degradation by isolated microorganisms. DMS was degraded by Thiobacillus sp. MS1 (5), and Hyphomicrobium sp. S (6). DMDS mixed with DMS was degraded by Thiobacillus sp. MS1 (5). There are no reports on the removal of methyl sulfides from contaminated air by identified bacteria. There are no examples of application of identified bacteria for the treatment of malodorous gas.

O,O-Dimethyl phosphorodithioate (DMDTP) is a precursor in the production of such organophosphorus pesticides as malathion, dimethoate and so on and is abundant in wastewater of pesticide manufacturers. DMDTP has an offensive mercaptan-like odor and presents an odor problem in the pesticide industry. It has been reported that dialkyl phosphates and their sulfur analogs are

very stable in biological systems (7,8). The only reported instances of DMDTP utilization by microorganisms have occurred when the compounds were the sole phosphorus sources (8). Papers on biological treatment of wastes from organophosphorus pesticide manufacturers (9-11) have not mentioned degradation of dialkyl phosphorodithioates.

The present study was undertaken to prevent malodorous air pollution caused by these sulfur compounds. The author isolated bacteria being able to oxidize these compounds and developed efficient deodorizing processes using those bacteria.

CHAPTER 1 Degradation of O,O-Dimethyl Phosphorodithioate
by Activated Sludge^{a)}

INTRODUCTION

DMDTP is contained in wastewater from pesticide manufacturers. Most of the manufacturers use activated sludge process to treat the wastewater. Therefore, the development of DMDTP degradation processes using activated sludge is most useful for the manufacturers.

The present study was designed to examine the degradation of DMDTP by activated sludge. The author presents the first direct and definitive evidence for degradation of DMDTP and some other organophosphates by microorganisms.

MATERIALS AND METHODS

Chemicals. The organophosphorus compounds used and abbreviations are described in Table 1. DMDTP ammonium salt was

Table 1. Organophosphorus Compounds

Abbreviation	Chemical name	Chemical formula
DMDTP	<u>O,O</u> -Dimethyl phosphorodithioate	$(\text{CH}_3\text{O})_2\text{P}(\text{S})\text{SH}$
DMTP	<u>O,O</u> -Dimethyl phosphorothioate	$(\text{CH}_3\text{O})_2\text{P}(\text{S})\text{OH}$
DEDTP	<u>O,O</u> -Diethyl phosphorodithioate	$(\text{C}_2\text{H}_5\text{O})_2\text{P}(\text{S})\text{SH}$
DMP	Dimethyl phosphate	$(\text{CH}_3\text{O})_2\text{P}(\text{O})\text{OH}$
MP	Monomethyl phosphate	$\text{CH}_3\text{OP}(\text{O})(\text{OH})_2$
DEP	Diethyl phosphate	$(\text{C}_2\text{H}_5\text{O})_2\text{P}(\text{O})\text{OH}$
EP	Monoethyl phosphate	$\text{C}_2\text{H}_5\text{OP}(\text{O})(\text{OH})_2$

obtained from Aldrich Chemical Co., Milwaukee, U.S.A. DMTP sodium salt was kindly supplied by Sumitomo Chemical Co., Osaka, Japan. DEDTP ammonium salt was purchased from Strem Chemicals Inc., Danvers, Mass, U.S.A. DMP, MP and the mixture of DEP and EP (3:7) were obtained from ICN K & K Laboratories, N.Y., U.S.A. All other chemicals were obtained from commercial sources.

Acclimation of activated sludge. Activated sludge was originally made from soil and sewage at Inage, Chiba, Japan and cultured in diluted corn steep liquor for many years in author's laboratory. The basal medium consisted of 200 mg of glucose, 200 mg of yeast extract and 500 mg of DMDTP in 1 liter of tap water, pH 7.0. The acclimation of the activated sludge was carried out by a fill-and-draw method in a batch system. A mixture of 1.5 liter of the basal medium and the activated sludge in 2.5 liter mini jar-fermentor was cultured with aeration at 30°C for 23 hr; aeration was stopped and after 30 min standing, 0.5 liter of the treated water, i.e. upper liquid phase, was replaced with fresh basal medium, and acclimation was continued.

Procedures for degradation test. The acclimated activated sludge was washed three times with tap water by decantation and suspended in tap water to a mixed liquor suspended solid concentration of 5000 mg/liter. To 1.5 liter portions of the mixed liquor in 2.5 liter mini jar-fermentors was added 500 mg of DMDTP per liter. The mixture was cultured with aeration at 30°C. The pH of the mixed liquor was maintained at 7.0 by suitable addition of 1 N-NaOH using pH controller HBR-92 (Denki Kagaku Keiki Co., Tokyo, Japan). At intervals, 15 ml

portions were removed and filtered through membranes of 0.45 μ m pore diameter to allow for analysis.

Analytical methods

Estimation of DMDTP. DMDTP was converted to the cupric complex which was soluble in carbon tetrachloride with the formation of an intense yellow color. The color intensity was proportional to the concentration of DMDTP and was measured colorimetrically at 420 nm.

Estimation of Dissolved total organic carbon (TOC). Dissolved TOC was estimated with a Beckman model 102A TOC analyzer.

Estimation of inorganic orthophosphate (P_i). P_i was estimated by the colorimetric method of molybdenum blue with the use of stannous chloride as a reducing agent (12).

Estimation of sulfate. Sulfate was precipitated as barium sulfate using barium chromate suspension in an acid solution, and excess barium was almost completely precipitated as barium chromate by adding ammonia water (containing calcium ion) and ethanol. Finally the amount of chromate which was exchanged with sulfate was estimated colorimetrically at 370 nm (13).

Estimation of mixed liquor suspended solids (MLSS). MLSS was determined by sludge weight dried at 105°C overnight after centrifugation at 3000 rpm for 10 min and washing.

Thin-layer chromatography (TLC). The degradation products in the extracellular solution were separated in TLC system. TLC plates (20 x 20 cm) of 0.25 mm layers of silica gel 60 (Merck, Art. 5721) were used. The solvent system was 2-propanol-28%

ammonium hydroxide (75:25, by volume) (14). The sulfur containing compounds were detected by spraying with 1% DQC(2,6-dibromobenzoquinone-4-chloroimide) in acetic acid (15). The phosphorus derivatives were located by the method of Hanes and Isherwood (16), using ultraviolet light as the reducing agent.

RESULTS

Acclimation of activated sludge

A drop in pH was observed in mixed liquor during acclimation to DMDTP, and the pH was adjusted to 7.0 by addition of 1 N-NaOH whenever the medium was replaced. The activated sludge did not degrade DMDTP to P_i by this acclimation method for three months. Then the acclimation was carried out using a pH controller to maintain the pH of the mixed liquor at 7.0. As shown in Fig. 1, DMDTP disappeared after 6 days, but at this point of time, a decrease in TOC or an increase in P_i was not observed. DMDTP was degraded to P_i after 9 days. This acclimated, activated sludge was able to degrade 500 mg/liter of DMDTP completely in a day. The following experiments concern the degradation of DMDTP and some other organophosphorus compounds by this sludge.

Degradation of DMDTP

Degradation of DMDTP caused a drop in pH, which inhibited further degradation (Fig. 2). Therefore controlling the pH of the mixed liquor was thought essential to degrade DMDTP. The effect of pH on DMDTP degradation was determined using pH controller. Figure 3 shows that 500 mg/liter of DMDTP was

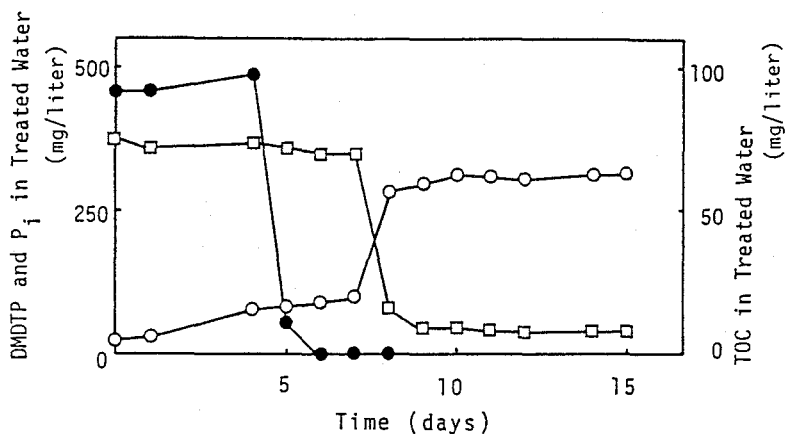


Fig. 1. Acclimation Behavior of Activated Sludge. The pH of mixed liquor was maintained at 7.0 ± 0.1 by a pH controller. MLSS concentration was 6000 mg/liter. As the sample of zero time, 10 ml portion of the mixed liquor was removed and filtered 10 min after the beginning of the acclimation test.

●, DMDTP; ○, P_i; □, TOC.

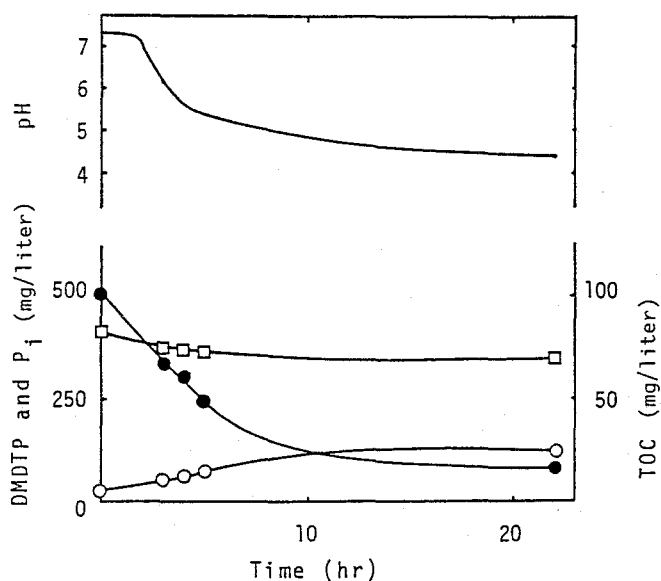


Fig. 2. Degradation Test of DMDTP without pH Control.

●, DMDTP; ○, P_i; □, TOC.

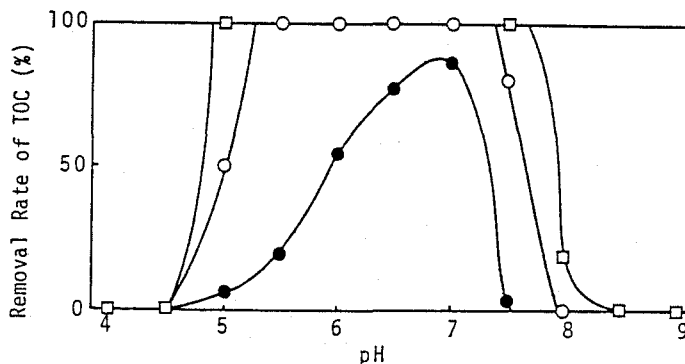


Fig. 3. Effect of pH on DMDTP Degradation.

Degradation test of 500 mg/liter of DMDTP was carried out at various pH. The pH of mixed liquor was adjusted and maintained at a fixed pH value by suitable additions of 1 N-NaOH and 1 N-HCl, using a pH controller.

●, after 4 hr; ○, after 8 hr; □, after 24 hr.

degraded to P_i in a day when the pH range was from 5.0 to 7.5, and optimally from 6.5 to 7.0. DMDTP degradation did not occur at pH lower than 4.5 or higher than 8.5.

The degradation rate of DMDTP was nearly proportional to the concentration of activated sludge up to 7000 mg-MLSS/liter (Fig.4).

A high concentration (up to 20 g/liter) of DMDTP in mixed liquor did not inhibit the degradation (Fig. 5).

Wastewater of pesticide manufacturers contained more than 20% NaCl. It was found that DMDTP degradation was completely inhibited by 1.5% NaCl (Fig. 6).

Time course of DMDTP degradation

Figure 7 shows that 500 mg/liter (2.9 mM) of DMDTP was completely degraded after 7 hr. As a result, 260 mg/liter

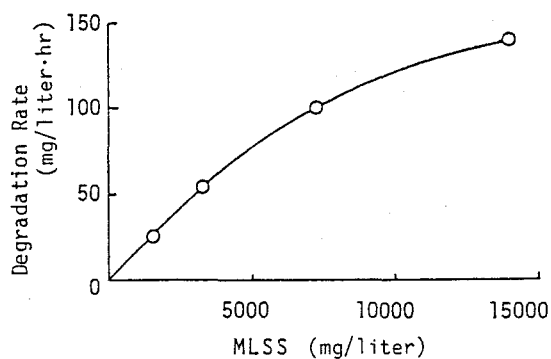


Fig. 4. Effect of Activated Sludge Concentration on DMDTP Degradation.

Degradation rate is the quotient of DMDTP concentration (500 mg/liter) by time (hr) required for the complete degradation.

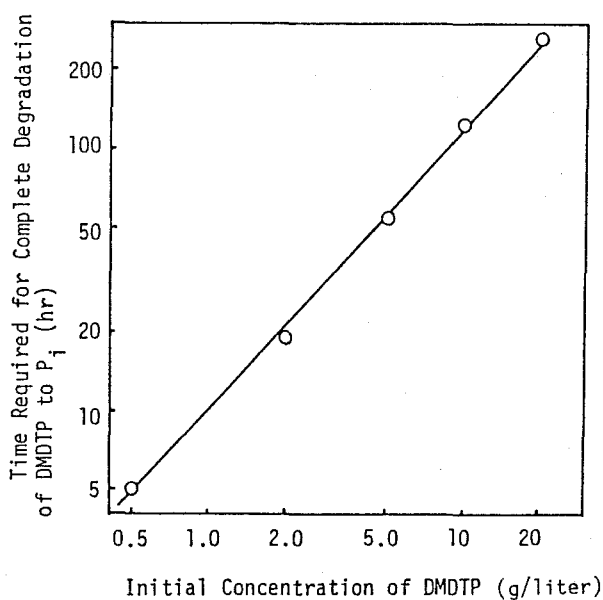


Fig. 5. Effect of Initial DMDTP Concentration.

Completion of DMDTP degradation was determined by estimation of TOC and P_i in extracellular solution.

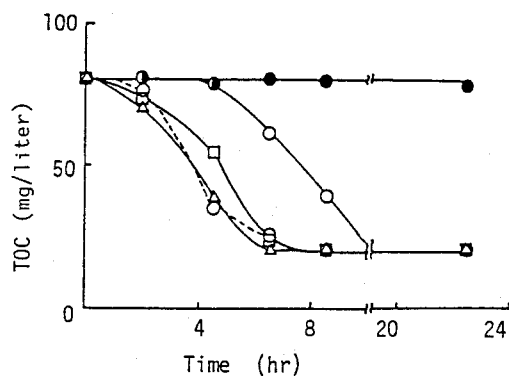


Fig. 6. Effect of NaCl on DMDTP Degradation.
NaCl concentration: ○-○, 0.02%; △-△, 0.2%; □-□, 0.5%;
○-○, 1.0%; ●-●, 1.5%.

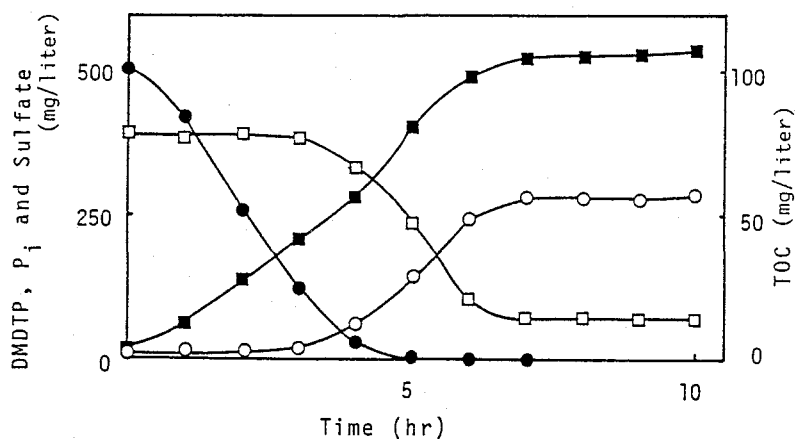


Fig. 7. Time Course of DMDTP Degradation.
●, DMDTP; ○, P_i; ■, sulfate; □, TOC.

(2.7 mM) of P_i and 510 mg/liter (5.3 mM) of sulfate were produced and 64 mg/liter (5.3 mM) of TOC was removed. For neutralization 680 mg/liter (17 mM) of sodium hydroxide was necessary. An increase in P_i occurred after 4 hr when almost all DMDTP disappeared. The decrease of TOC coincided with the increase of P_i.

Samples from the degradation test were separated on TLC

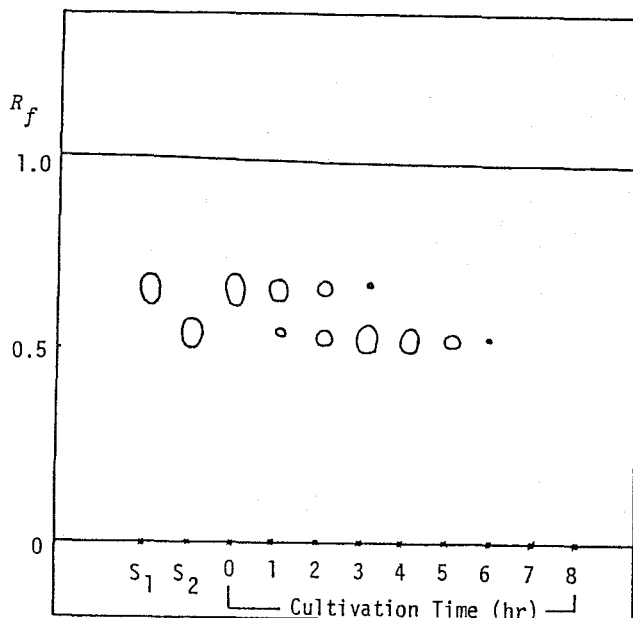


Fig. 8. TLC of Extracellular Solution from the Degradation Test of DMDTP.

Splay reagent was 1% DQC in acetic acid.

S₁, DMDTP; S₂, DMTP.

plates, and sulfur-containing compounds were located (Fig. 8). DMTP was detected as an intermediate product of DMDTP degradation. Then phosphorus-containing compounds were located. DMTP was found to be the only intermediate product.

Degradation test of other organophosphorus compounds

Wastewater of pesticide manufacturers contained various kind of organophosphorus compounds. Degradation of MP, DMP, DMTP, DEP-EP mixture and DEDTP was examined. The activated sludge acclimated to DMDTP degraded all of them (Fig. 9), while that cultured in diluted corn steep liquor did not degrade any of them. The organophosphorus compounds without sulfur were degraded much faster than those with sulfur.

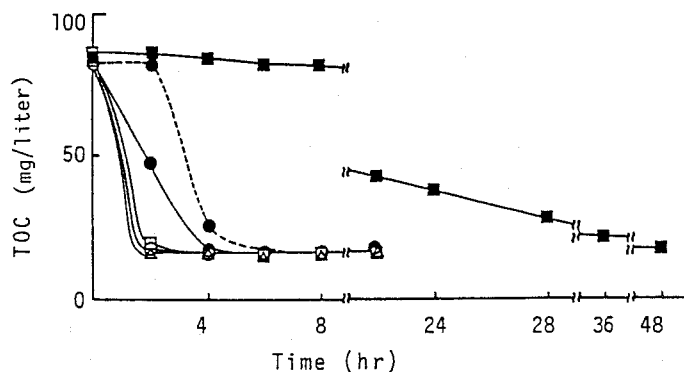


Fig. 9. Degradation of Organophosphorus Compounds by Activated Sludge Acclimated to DMDTP.

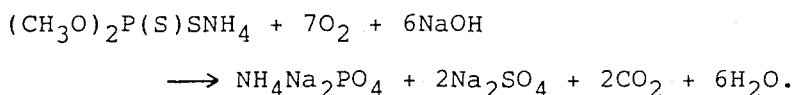
To the activated sludge each organophosphate was added at 70 mg-C/liter. The pH was maintained at 7.0.

●-●, DMDTP; ●-●, DMTP; ■-■, DEDTP; ○-○, DMP; □-□, DEP-EP mixture; △-△, MP.

The acclimation method using a pH controller was adopted to degrade these organophosphorus compounds. Activated sludge degraded each compound to P_i by acclimation within three weeks.

DISCUSSION

The results indicate that DMDTP and some other organophosphates are not stable to attack by acclimated activated sludge which is a mixture of various microorganisms. Quantitative analysis of DMDTP degradation shows that the degradation of 1 molecule of DMDTP caused the removal of 2 molecules of organic carbon, and the production of 1 molecule of inorganic phosphate and 2 molecules of sulfate, and required 6 molecules of NaOH for neutralization. The overall reaction, therefore, can be represented by the formula.



The degradative pathway of DMDTP by activated sludge is $\text{DMDTP} \rightarrow \text{DMTP} \rightarrow \text{DMP} \rightarrow \text{MP} \rightarrow \text{P}_i$, because (i) the intermediate product of DMDTP degradation was only DMTP, (ii) the activated sludge acclimated to DMDTP had the ability to degrade MP and DMP much faster than DMDTP and DMTP, and (iii) the decrease of TOC coincided with the increase of P_i .

In degrading DMDTP the control of pH in mixed liquor is very important. As wastewater of pesticide manufacturers does not contain a buffer, a drop in pH is likely to be caused by acids produced in the process of degradation and inhibits further breakdown. Excessive additions of alkali also inhibit the degradative reaction. The careful control of pH is essential for adequate treatment of wastewater.

SUMMARY

The activated sludge was acclimated to DMDTP by the fill-and-draw method. The acclimated activated sludge degraded 500 mg/liter of DMDTP in 7 hr and as a result, produced 260 mg/liter of P_i and 510 mg/liter of sulfate. The pH of the mixed liquor was adjusted to degrade DMDTP, and the optimum pH was from 6.5 to 7.0. The activated sludge acclimated to DMDTP degraded DMTP, DMP, DEDTP and DEP.

CHAPTER 2 Degradation of O,O-Dimethyl Phosphorodithioate
by Thiobacillus thioparus TK-1 and Pseudomonas
AK-2^{b)}

INTRODUCTION

DMDTP was degraded to P_i by acclimated activated sludge as described in Chapter 1. This chapter deals with the isolation of DMDTP-degrading bacteria from the activated sludge. The author isolated two bacteria, Thiobacillus thioparus and Pseudomonas sp. By a symbiont of these two bacteria, DMDTP was utilized as a sole energy and carbon source and was degraded to P_i .

MATERIALS AND METHODS

Chemicals. DMDTP used in degradation tests was purified by preparative layer chromatography as follows. One milliliter of 50% DMDTP was streaked across the width of a chromatoplate of a 2-mm layer of silica gel 60 F₂₅₄ (Merck, Art. 5717). After development in a solvent system of acetonitrile-water (95:5, by volume), DMDTP was visualized by ultraviolet light (2536 Å). The band of DMDTP was transferred onto filter paper and DMDTP was eluted from the silica gel with distilled water.

All other chemicals were used without further purification.

Medium. The trace metal solution consisted of 50.0 g of ethylenediamine tetraacetic acid, 22.0 g of $ZnSO_4 \cdot 7H_2O$, 5.54 g of $CaCl_2$, 5.06 g of $MnCl_2 \cdot 4H_2O$, 4.99 g of $FeSO_4 \cdot 7H_2O$, 1.10 g of

$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 1.57 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 1.61 g of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ in 1 liter of distilled water and the pH was adjusted to 6.0 with KOH.

The vitamin mixture consisted of 10 mg of thiamine·HCl, 20 mg of nicotinic acid, 20 mg of pyridoxine·HCl, 10 mg of p-aminobenzoic acid, 20 mg of riboflavin, 20mg of calcium pantothenate, 1 mg of biotin and 1 mg of cyanocobalamin in 1 liter of distilled water, pH 7.0.

Basal medium A consisted of 4 g of KH_2PO_4 , 4 g of K_2HPO_4 , 0.4g of NH_4Cl , 0.08 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1 ml of the trace metal solution in 1 liter of distilled water. Medium AS and AP were prepared by adding 2-4 g of DMDTP and 5 g of DMP, respectively, to 1 liter of basal medium A.

Basal medium B consisted of 2 g of KH_2PO_4 , 2 g of K_2HPO_4 , 0.4 g of NH_4Cl , 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 2 ml of the trace metal solution in 1 liter of distilled water. Medium BVS and BCS were prepared by adding 5 ml of the vitamin mixture plus 1 g of DMDTP and 1 g of Na_2CO_3 plus 0.5 g of purified DMDTP, respectively, to 1 liter of basal medium B. All media were adjusted to pH 7 with NaOH.

Agar slants and agar plates were prepared by addition of agar in a proportion of 2% to the medium. Silica gel slants and silica gel plates were prepared as follows. Medium BVS of ten-fold concentration, colloidal silica (Snowtex, Nissan Chemical Industries Ltd., Tokyo, Japan) and 6% HCl were sterilized separately at 120°C for 15 min in an autoclave. Then, 90 ml of the colloidal silica was neutralized by the addition of 4 ml of

6% HCl and mixed with 10 ml of the medium. To prepare silica gel slants, 6 ml portions of the mixture were replaced in test tubes (diameter: 16.5 mm). To prepare silica gel plates, 20 ml portions of the mixture were replaced in petri dishes. The mixture solidified after about 20 hr at room temperature.

Enrichment culture. Activated sludge cultured in diluted corn steep liquor was acclimated to DMDTP as described in Chapter 1. To a 0.5 liter portion of the acclimated activated sludge in a 2.5-liter mini jar-fermentor was added 0.5 liters of 0.2% DMDTP in tap water. The mixed liquor was cultured with aeration at 30°C. The pH of the mixed liquor was maintained at 7.0 by suitable addition of 1 N-NaOH using a pH controller. After 24 hr and 48 hr, 50 ml of 2% DMDTP was added. After 72 hr, 0.5 liter of the mixed liquor was replaced with 0.5 liters of 0.2% DMDTP in tap water and the cultivation was continued. This process was repeated three times. Then the mixed liquor was centrifuged (10,000 rpm for 15 min at 5°C). The pellet was washed twice with 0.85% NaCl and suspended in 0.85% NaCl. The suspension was inoculated to medium AS, cultured with aeration at 30°C for 10 days and then inoculated to fresh medium AS. This process was repeated several times.

Analytical methods. Analytical methods to estimate DMDTP, dissolved TOC, P_i and sulfate were the same as described in Chapter 1. Protein was estimated by the method of Lowry et al. (17).

Thin-layer chromatography (TLC). The degradation products in the extracellular solution were separated in a TLC

system. TLC plates of a 0.25 mm layer of silica gel 60 (Merck, Art. 5721) were used. Solvent system A was 2-propanol-28% ammonium hydroxide (75:25, by volume) (14). Solvent system B was acetonitrile-water (88:12, by volume) (15). The sulfur containing compounds were detected by spraying with 1% DQC (2,6-dibromobenzoquinone-4-chloroimide) in acetic acid (15). The phosphorus derivatives were located by the method of Marcus et al. (18).

GC content of DNA. DNA was extracted by the method of Marmur (19), and the base composition was calculated from its thermal denaturation temperature.

Coenzyme Q system. Quinone was extracted according to the method of Yamada et al. (20) with some modification as described by Katayama-Fujimura and Kuraishi (21), and was analyzed by paper chromatography and mass spectrometry.

Analysis of fatty acids. The cellular fatty acids were extracted from lyophilized cells after saponification, methylated with diazomethane, and then tentatively identified by gas-liquid chromatography both in the polar and nonpolar liquid phase as described by Katayama and Kuraishi (22). Nonpolar and hydroxy fatty acids were separated by thin-layer chromatography, and the corresponding layers were scraped off and eluted with ether. Bromination and refluxing with methanolic hydrogen chloride were carried out to identify unsaturated and cyclopropane fatty acids (22).

RESULTS

Enrichment culture

Medium AS inoculated with the enrichment culture became turbid with yellowish white granules supposed to be elemental sulfur after incubation at 30°C for one or two days. The granules disappeared gradually and the medium turned pink. The enrichment culture apparently consisted of at least two strains of bacteria, one with a reddish pigment and one with no pigment.

Isolation of strains

The resulting enrichment culture was streaked on agar plates of medium BVS. The plates were incubated at 25°C. Very small colonies appeared after 10 days. These colonies did not grow bigger and were too small to get pure cultures.

Then the resulting enrichment culture was streaked on silica gel plates of medium BVS. Small colonies (0.5 mm in diameter) appeared after incubation at 25°C for 5 days. There were no colonies with a reddish pigment. Each colony was inoculated into a test tube (16.5 mm in diameter) containing 2 ml of medium BVS. Cultivation was aerobically carried out at 25°C for a week. The cultures were restreaked on silica gel plates of medium BVS. Strain TK-1 grew best and was used in the following experiments.

Utilization of DMDTP

Strain TK-1 was aerobically cultured at 30°C for 72 hr in medium BCS containing DMDTP as a sole energy and organic carbon source. The medium turned slightly turbid. The pH dropped from 7.25 to 6.60. DMDTP in the medium disappeared completely and 480

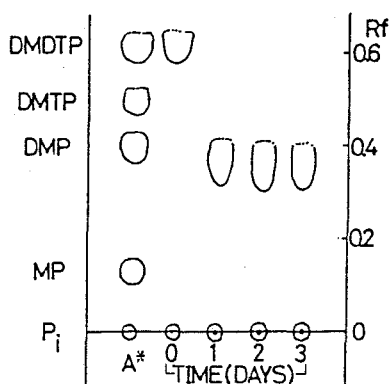


Fig. 10. TLC of Extracellular Solution from the Utilization Test of DMDTP by Strain TK-1.

Strain TK-1 was aerobically precultured at 25°C for 72 hr in 10 ml of medium BCS and inoculated to 200 ml of medium BCS. Cultivation was aerobically carried out at 30°C. An aliquot (40 μ l) of the culture was developed using solvent system A. The phosphorus containing compounds were detected.

A*, authentic compounds.

mg/liter of sulfate (1.75 mol per mol of consumed DMDTP) were produced. TOC in the extracellular solution, however, did not decrease. The degradation products were then separated with a TLC system and phosphorus-containing compounds were located (Fig. 10). The R_f values of the product were the same as those of DMP in both solvent system A and B. The product was not considered to contain sulfur because almost all sulfur provided as DMDTP was detected as sulfate in the extracellular solution and, moreover, the product gave no color by spraying with 1% DQC. Therefore the product was concluded to be DMP. By this experiment, it was revealed that strain TK-1 utilized DMDTP as an energy source, not as a carbon source, and that strain TK-1 oxidized DMDTP to sulfate and DMP.

Other isolates were examined to determine whether they degraded DMDTP to P_i . All isolates could only desulfurize DMDTP, and produced sulfate and DMP.

Isolation of DMP-degrading bacteria

The resulting enrichment culture was streaked on agar plates of medium AP and incubated at 25°C for a week. Each colony was inoculated to a test tube containing 5 ml of medium AP and cultured aerobically at 25°C for a week before restreaking on an agar plate of medium AP. This procedure was repeated four times. Colonies of all isolates had a reddish pigment. No isolates could grow in media containing DMDTP as a sole energy and organic carbon source. Strain AK-2 grew best in medium AP and was used in the following experiments.

Degradation of DMDTP by strain TK-1 and strain AK-2

Degradation tests were carried out using the medium containing 0.5 g/liter of purified DMDTP as a sole energy and organic carbon source.

Experiment I. Strain TK-1 was inoculated to the medium and cultivated. After 3 days, when DMDTP was desulfurized to DMP, strain AK-2 was inoculated. As shown in Figs. 11 and 12, DMDTP completely disappeared in 3 days with the growth of strain TK-1. Strain TK-1 produced DMP and 504 mg/liter of sulfate (1.84 mol of sulfate per mol of DMDTP consumed). In the 2-day broth, a slight amount of an intermediate product of DMDTP desulfurization by strain TK-1 was detected on a TLC plate (Fig. 12). The R_f value of the product was the same as that of DMTP in both solvent system A and B. By spraying with 1% DQC, the product gave a

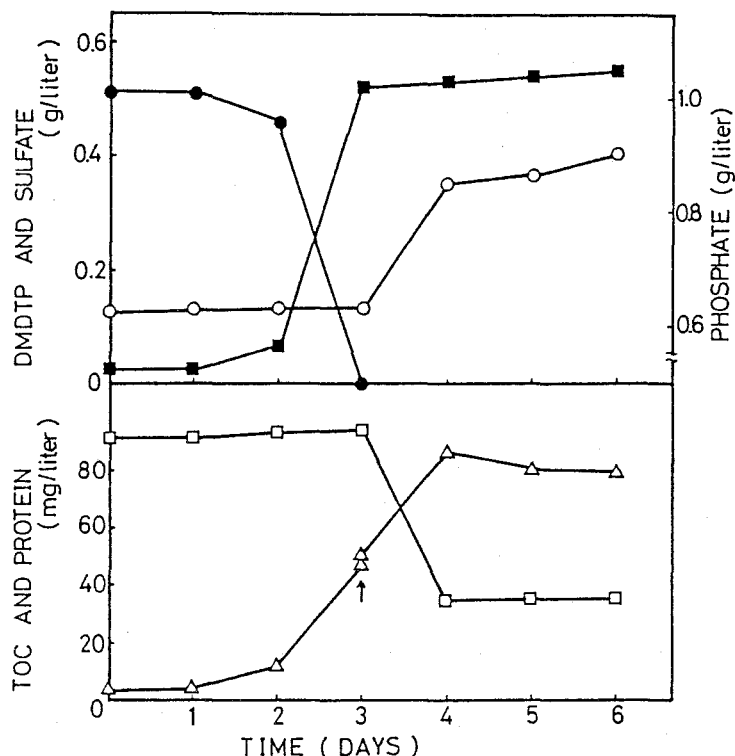


Fig. 11. Degradation of DMDTP (Experiment I).

The bacterial cells of strain TK-1 prepared from a 3-day preculture at 30°C in 200 ml of medium BCS were collected, washed and suspended in 15 ml of 0.85% NaCl. The cell suspension was inoculated into a 2.5-liter mini jar-fermentor containing the medium which consisted of 0.5 g of purified DMDTP, 0.5 g of KH_2PO_4 , 0.5 g of K_2HPO_4 , 0.05 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.004 g of NH_4Cl , 0.125 g of Na_2CO_3 , 0.5 ml of trace metal solution and 1 liter of distilled water. Cultivation was aerobically carried out at 30°C. The pH of the medium was maintained at pH 6.8 by suitable addition of 2% Na_2CO_3 using a pH controller. After 3 days, the cell suspension of strain AK-2 prepared from a slant culture was inoculated to the medium as indicated by the arrow, and the cultivation was continued.

●, DMDTP; ■, sulfate; ○, P_i ; □, TOC; Δ, protein.

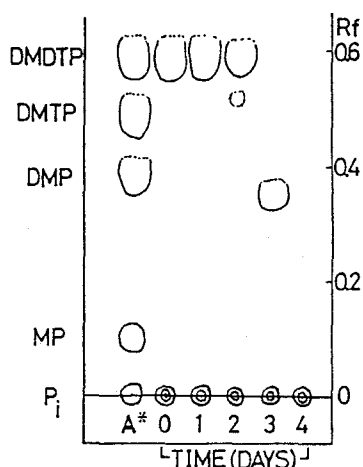


Fig. 12. TLC of Extracellular Solution from Experiment I. Solvent system A was used. The phosphorus containing compounds were detected.
A*, authentic compounds.

reddish color. Therefore the product was concluded to be DMTP. DMP, produced from the desulfurization of DMDTP by strain TK-1, was degraded by strain AK-2 and 220 mg/liter of P_i (0.81 mol per mol of DMDTP consumed) were produced. The decrease in TOC coincided with the production of P_i.

Experiment II. The bacteria cells of strain TK-1 and strain AK-2 prepared from the 7-day culture in Experiment I were collected, washed and then inoculated to medium BCS. DMDTP was completely degraded to P_i by bacterial symbiosis involving strain TK-1 and strain AK-2 in 40 hr (Figs. 13 and 14). An intermediate product was detected on a TLC plate (Fig. 14) and was concluded to be DMTP by the R_f value in the two solvent systems and the reddish color given by spraying with 1% DQC. DMP was not detected in the medium.

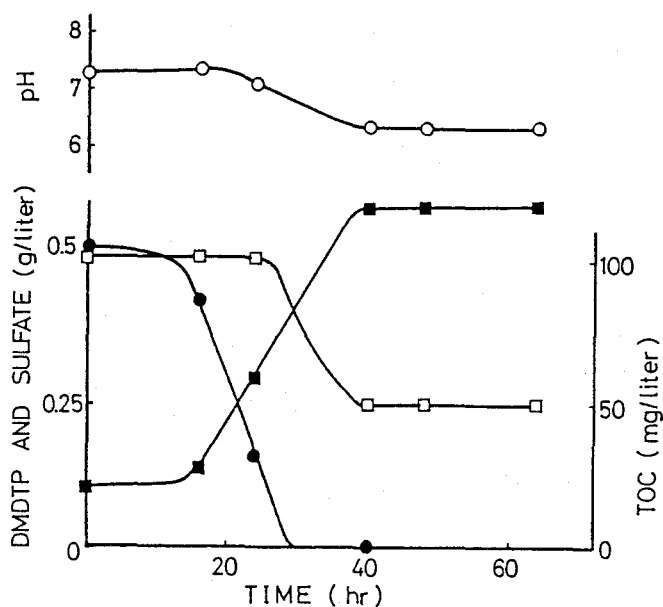


Fig. 13. Degradation of DMDTP (Experiment II). The bacterial cells of strain TK-1 and strain AK-2 prepared from 500 ml of the 7 day culture in Experiment I were collected, washed and inoculated into a 300-ml Erlenmeyer flask containing 100 ml of medium BCS. Cultivation was aerobically carried out at 30°C. ●, DMDTP; ■, sulfate; □, TOC.

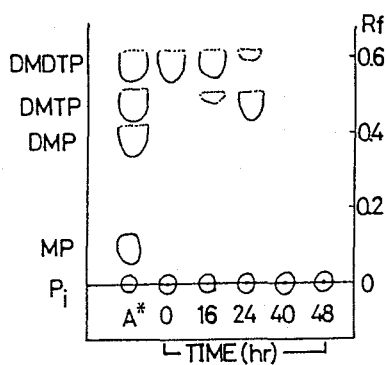


Fig. 14. TLC of Extracellular Solution from Experiment II. Solvent system A was used. The phosphorus containing compounds were detected. A*, authentic compounds.

Taxonomical characteristics of strain TK-1

Strain TK-1 was a Gram-negative rod (0.4 by 1.0-1.5 μ m), motile with a polar flagellum (Fig. 15). Colonies on thiosulfate agar were small (1 mm in diameter), circular and yellowish white due to precipitated sulfur. Strain TK-1 could grow with thiosulfate and the pH of the medium dropped to 4.85. Tetrathionate was not accumulated in the medium.

Strain TK-1 was able to grow in an autotrophic medium containing elemental sulfur or tetrathionate, but was not able to grow in heterotrophic media such as nutrient broth and the broth supplemented with yeast extract and/or glucose.

Nitrate was reduced to nitrite, but nitrogen was not produced. The G+C content of DNA was 62.1 mol%. The cellular fatty acid composition consisted of saturated straight chains of C₁₀, C₁₂, C₁₆ and C₁₈, mono-unsaturated straight chains of C₁₆ and C₁₈, a cyclopropane acid of C₁₇, and 3-hydroxy fatty acids of C₁₀ and C₁₂. The main components were C_{16:0} and C_{16:1}.

The optimum pH was between 6.5-7.0. Growth occurred between pH 5.0-8.0. The optimum temperature was 30°C. Growth occurred between 10-40°C.

From these characteristics, strain TK-1 was identified as Thiobacillus thioparus on the basis of the description in Bergey's Manual of Determinative Bacteriology, 8th Ed. (23). Although the composition of the cellular fatty acids was not the same as that of T.thioparus described in Bergey's Manual, it was identical with that of T.thioparus reported by Katayama and Kuraishi (22).

The usefulness of the coenzyme Q system in bacteria taxonomy was pointed out in the review of Collins and Jones (24). Strain TK-1 had an eight isoprene ubiquinone (Q₈), and this result agrees with those of Cook and Umbreit (25), and Kuraishi et al. (26).

Taxonomical characteristics of strain AK-2

Taxonomical characteristics of strain AK-2 were as follows: Cells were rods (0.8-1.0 by 1.5-2.5 μ m) and motile with a polar flagellum (Fig. 16). Gram-negative. Not acid-fast. Not spore forming. Colonies on a bouillon agar plate containing phosphate buffer were moderate in growth, circular, wrinkled, flat with a slightly raised center, and pink with a red center. Colonies on an agar plate containing methanol were moderate in growth, entire, flat, pink and opaque. Liquefaction of gelatin was negative. Litmus milk was not changed. Nitrate was reduced to nitrite, but nitrogen was not produced. Methyl red test negative. Voges-Proskauer test negative. Indole was not produced. Hydrogen sulfide was not produced. Starch hydrolysis negative. Water soluble pigment was not produced. Poly- β -hydroxybutyrate was accumulated. Urease positive. Catalase positive. Oxidase positive. Citrate was utilized. Nitrates and ammonium salts were utilized as nitrogen sources. Acids were produced from D-fructose, D-xylose and glycerol. Acids were not produced from D-glucose, D-mannose or maltose. Organic growth factors were not required. The G+C content of DNA was 69.6 mol%. Aerobic. Optimum temperature was 30°C. Growth occurred between 15-37°C. Optimum pH was 6.5. Growth occurred between pH 5-8.



Fig. 15. Electron Micrograph of Strain TK-1.
The cells of strain TK-1 were used in the early phase of growth on the medium containing thiosulfate. For electron microscopy, the bacterial cells were negatively stained with 0.5% phosphotungstic acid.

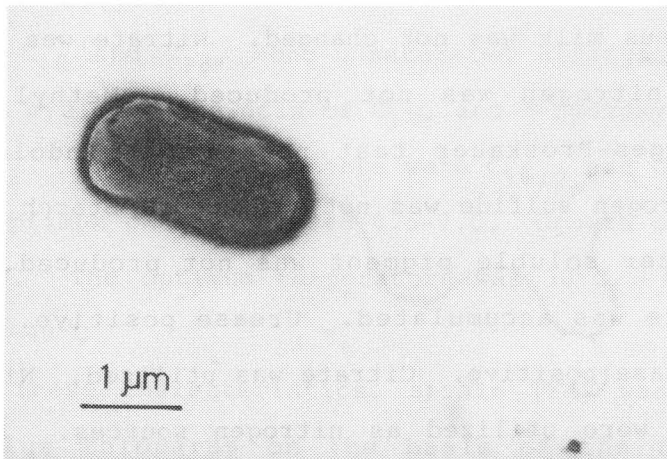


Fig. 16. Electron Micrograph of Strain AK-2.
The cells of strain AK-2 were used in the early phase of growth on the medium containing methanol. For electron microscopy, the bacterial cells were negatively stained with 0.5% phosphotungstic acid.

Table 2. Assimilation of Carbon Compounds by Strain AK-2.

Assimilation of carbon compounds was tested by using basal medium B at 25°C for 14 days. The concentration of carbohydrates and alcohols was 5 mg/ml. That of formaldehyde and formamide was 0.2 mg/ml. That of the other carbon compounds was 2 mg/ml.

Carbon source	Growth*	Carbon source	Growth*
Methanol	++	<u>D</u> -Sorbitol	-
Formaldehyde	-	<u>L</u> -Rhamnose	-
Formate	+	<u>D</u> -Xylose	±
Monomethylamine	+	<u>L</u> -Arabinose	-
Dimethylamine	-	<u>D</u> -Glucose	±
Formamide	-	<u>D</u> -Galactose	-
Ethanol	++	<u>D</u> -Mannose	±
1-Propanol	+	<u>D</u> -Fructose	++
Acetate	++	Sucrose	-
Propionate	++	Lactose	-
Oxalate	+	Trehalose	-
Lactate	++	Cellobiose	-
Pyruvate	++	Maltose	±
Glycolate	±	Glycerol	++
Glyoxylate	±	Starch	-
Malonate	+	<u>L</u> -Alanine	++
Maleate	+	<u>L</u> -Leucine	-
<u>L</u> -Malate	++	<u>L</u> -Serine	-
Fumarate	++	<u>L</u> -Lysine	-
<u>DL</u> -Tartarate	++	<u>L</u> -Aspartate	++
Succinate	++	<u>L</u> -Glutamate	++
Glutarate	±	Glycine	-
Citrate	++	p-Aminobenzoate	-
Gluconate	+		

* ++, abundant growth; +, moderate growth;
±, weak growth; -, no growth.

These results indicate that strain AK-2 belongs to the genus Pseudomonas according to Bergey's Manual, 8th Ed.¹⁴⁾ Strain AK-2 is a facultative methylotroph because this strain utilized methanol and other organic compounds as sole carbon sources (Table 2). The coenzyme Q system of strain AK-2 was Q₁₀. The cellular fatty acid composition consisted of saturated straight chains of C₁₆, C₁₇ and C₁₈, mono-unsaturated straight chains of C₁₆ and C₁₈, and cyclopropane acids of C₁₇ and C₁₉. The major component was C_{18:1}.

Many Gram-negative methanol-utilizing bacteria have been isolated and characterized. Recently, Urakami and Komagata (27) reported detailed taxonomic studies on these bacteria on the basis of morphological characteristics, cellular fatty acid composition and the coenzyme Q system, and divided the bacteria into four groups. Strain AK-2 is included in group 2. However, a classification system for these bacteria has not been established. Therefore, the taxonomical position (species) of strain AK-2 cannot be defined at present.

DISCUSSION

Two bacteria were isolated from activated sludge. One of them, strain TK-1 which was identified as Thiobacillus thioparus, grew in a medium containing DMDTP as a sole energy source. This bacterium desulfurized DMDTP and produced DMP and sulfate. The other, strain AK-2 which was identified as Pseudomonas sp., grew in a medium containing DMP as a sole energy and carbon source,

and degraded DMP to P_i . This is the first definite evidence for the existence of a bacterium able to utilize DMDTP as a sole energy source and a bacterium able to utilize DMP as a sole energy and carbon source. By the symbiotic action of the two bacteria, DMDTP was utilized as a sole energy and carbon source, and was degraded to P_i . Inhibition of growth by one of the two bacteria of the other could not be observed in the degradation tests.

In Chapter 1, the author showed that DMDTP was degraded to P_i by activated sludge and supposed that the degradation pathway was $\text{DMDTP} \rightarrow \text{DMTP} \rightarrow \text{DMP} \rightarrow \text{MP} \rightarrow P_i$. The isolation of the two bacteria here suggests that this pathway actually occurs.

SUMMARY

Two bacteria were isolated from activated sludge able to degrade DMDTP. One of them, strain TK-1 identified as Thiobacillus thioparus, utilized DMDTP as a sole energy source and produced DMP and sulfate. The other, strain AK-2 identified as Pseudomonas sp., utilized DMP as a sole energy and carbon source and degraded DMP to P_i . DMDTP was degraded to P_i by the co-action of the two bacteria.

CHAPTER 3 Degradation of Dimethyl Sulfide by Mixed Cultures and by Thiobacillus thioparus^{c)}

INTRODUCTION

DMDTP was degraded by the concerted action of two bacteria, Pseudomonas sp. AK-2 and T.thioparus TK-1 as described in Chapter 2. The latter grew at the expense of the sulfide oxidation, producing DMP and sulfate, while the Pseudomonas used DMP for growth, releasing P_i .

The aim of the present work was to establish whether DMS could be used as a substrate by these bacteria, either singly or in mixed culture, and to see whether pure cultures of bacteria capable of DMS oxidation could be obtained from activated sludge acclimated to DMS.

MATERIALS AND METHODS

Organisms and culture conditions. The isolation of T.thioparus TK-1 and Pseudomonas sp. AK-2 was described in Chapter 2. T.thioparus TK-1 and the derived strain TK-m, were maintained on agar slants containing 25 mM $Na_2S_2O_3$. Pseudomonas sp. AK-2, and the derived strain AK-m, were maintained on agar slants containing 0.2%(w/v) DMP.

Culture (50 ml) were grown in 300-ml erlenmeyer flasks sealed with rubber 'Suba seal' stoppers and shaken at 30°C, unless otherwise described.

Media. Basal medium B2 contained 2 g of KH_2PO_4 , 2 g of K_2HPO_4 , 0.4 g of NH_4Cl , 0.4 g of Na_2CO_3 , 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 2 ml trace metal solution (28) in 1 liter of distilled water. Medium B2V was medium B2 supplemented with 5 ml vitamin mixture (described in Chapter 2) per liter. Low-sulfate Basel Medium C was the same as medium B2V, except that it contained 0.2 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ instead of magnesium sulfate, and only 1 ml trace metal solution per liter.

Agar plates and slants were prepared by adding substrate and agar (1.5% w/v) to Medium B2V. Silica gel plates were prepared as follows: 90 ml of colloidal silica (Snowtex 20: Nissan Chemical Industries Ltd., Tokyo, Japan) were sterilized at 120°C (10 min), cooled, supplemented with a separately sterilized solution (6 ml) containing KH_2PO_4 (0.2g), K_2HPO_4 (0.2g) and Na_2CO_3 (0.04 g), then neutralized with about 2.2 ml of 1.7 N HCl (167 ml conc. HCl/liter). The following presterilized solutions were then added: 1 ml 4%(w/v) NH_4Cl , 0.5 ml 4%(w/v) $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 ml trace metal solution, 0.5 ml vitamin mixture, and 0.01 or 0.05 ml of unsterilized DMS. The medium (25 ml portions) was dispensed into petri dishes which were sealed with masking tape. The mixture solidified after 2-3 days at room temperature.

Chemostat cultures. A chemostat culture (960 ml) of T. thioparus TK-1 and Pseudomonas sp. AK-2 was established in a glass fermentor vessel (LH Engineering) with stirring (800 rev./min) and aeration. The culture was maintained at 30°C and at pH 6.7 by automatic titration with 0.25 M K_2CO_3 . The inoculum culture was grown in flasks on Medium B2 containing 5.7 mM DMDTP.

After inoculation (15% v/v), the culture was allowed to establish by batch growth on 2.9 mM DMDTP before continuous culture was commenced using medium B2 containing 4.4 mM DMDTP.

Measurement of DMS oxidation by *T.thioparus* TK-m. Oxygen uptake was measured using a teflon-covered Clark oxygen electrode cell (Rank Bros., Cambridge, U.K.) linked to a chart recorder. *T.thioparus* TK-m was grown (50-ml cultures in 9 sealed flasks) on medium C containing 1.4 mM DMS. DMS at 1.4 mM was added after 48 and 72 hr and then at 2.8 mM after 79 hr. After 96 hr (A_{660} , 0.205), organisms were harvested by centrifuging (17700 xg, 5°C, 10 min), washed once with 0.026 M potassium phosphate (pH 6.8) and resuspended in 3 ml of the same buffer. Oxygen uptake was measured at 30°C in the phosphate buffer (final volume, 3 ml) containing 0.01-0.3 mM DMS. The reaction was initiated by injecting 0.1 ml of cell suspension with a syringe.

Analytical methods. Growth of cultures in sealed flasks on DMS was monitored by removal of samples by syringe and measuring A_{660} in a Unicam SP1700 or LKB spectrophotometer. TOC was estimated by centrifuging 4-ml samples of cultures and assaying TOC in the supernatant liquids and pellets. TOC in the pellets was determined after suspending them in 0.025 M KH_2PO_4 , and represented cell-carbon. TOC was measured with a Beckman 915-B TOC analyzer. DMS in culture samples (0.2-0.5ml) was extracted with 5 ml 2,2,4-trimethylpentane and 2 ml of this solution was mixed with 2 ml 0.2% (w/v) iodine in trimethylpentane. DMS was estimated from the height of the peak at 300 nm. Sulfate was determined turbidimetrically as barium sulfate

and by using atomic absorption spectrophotometry to measure residual barium following sulfate precipitation. Methanol was measured with a Pye 104 gas chromatograph.

To measure $^{14}\text{CO}_2$ -incorporation by T.thioparus TK-m growing in sealed flasks with DMS, cultures were supplemented with 5 mM $\text{NaH}^{14}\text{CO}_2$ in medium C from which Na_2CO_3 was omitted. Samples (2 ml) were filtered through Whatman membrane filters (0.2 μm) and, after washing and drying, were counted in a PPO/POPOP scintillant (29) using a Beckman LS-7000 spectrometer.

Special chemicals. DMS was obtained from Aldrich Chemical Co. DMP (1:1 mixture with MP) was obtained from Strem Chemicals Inc., Newburyport, MA, U.S.A. $\text{Na}_2[^{14}\text{C}]\text{O}_3$ was from The Radiochemical Center (Amersham International).

RESULTS

Degradation of DMS by a chemostat culture of a mixture of T. thioparus TK-1 and Pseudomonas sp. AK-2

A steady-state co-culture was established at a dilution rate of 0.014 hr^{-1} on 4.4 mM DMDTP. DMS (2.8 mM) was then added to the medium feed and culture continued for 29 days. Following some initial perturbation, the steady state after 29 days exhibited the same turbidity (A_{660} , 0.3) and rate of acid production as that prior to DMS addition. A sample (5 ml) of the culture was inoculated into 45 ml medium C, with or without 1.4 mM DMS. After 5 days the culture with DMS showed an A_{660} of 0.09 and pH 6.87, compared with 0.044 and pH 7.07 for the culture

without DMS. Addition of a further 1.4mM DMS raised A_{660} to 0.152 (pH 6.67) after 24 hr, and another addition produced 0.20 (pH 6.38) in 24 hr. This culture was successfully taken through serial subculture (10% v/v inocula) on 1.4 mM DMS at 3 to 4-day intervals. After 15 such transfers, plate counts showed relative numbers of the organisms to be 100 TK-1 to 15 AK-2.

Characterization of bacteria growing on DMS in batch culture

The batch culture obtained above was streaked onto agar plates of medium C and the plates were incubated in an atmosphere containing DMS. It was also streaked onto silica-gel plates containing 1.4 or 7.0 mM DMS. No colonies were produced on these plates.

The culture was also streaked on plates of medium B2 containing either 25 mM thiosulfate or 16 mM DMP. Yellowish-white colonies containing precipitated sulfur developed on the thiosulfate plates: these were characteristic of T.thioparus TK-1. Pink colonies, as expected for Pseudomonas sp. AK-2, developed on the DMP plates. Each medium appeared to support only one colony type, equivalent to the two species initially used to inoculate the chemostat.

The largest colonies from each medium were picked off and subcultured on the same media and tested for ability to use DMS. They were coded T.thioparus TK-m and Pseudomonas sp. AK-m. Colonies of strain TK-m on thiosulfate agar were small (1 mM), circular and yellowish-white due to precipitated sulfur. In liquid medium strain TK-m grew autotrophically on thiosulfate, reducing the medium to about pH 5. No growth occurred on

nutrient agar, glucose + yeast extract agar or a yeast extract broth. Strain TK-m thus exhibited all the properties characteristic of T.thioparus TK-1 and was concluded to be a strain of the original culture.

Degradation of DMS by pure cultures

Strain TK-m was inoculated from thiosulfate agar into medium C containing 1.4 mM DMS. Degradation, accompanied by growth and drop in pH, was complete in 5 days. In contrast, strain TK-1 taken from thiosulfate took 12 days to develop to the same extent. Addition of further DMS (1.4 mM) to either culture resulted in its degradation within 24 hr, indicating development of the ability by both organisms to grow rapidly on DMS.

A batch culture of strain TK-m (200 ml in a 500-ml flask) on 1 mM DMS showed little variation in total organic carbon in the culture during growth, although DMS was completely consumed and 0.9 mM sulfate was produced (Fig. 17). Addition of a further 2 mM DMS was followed by a similar pattern of growth with disappearance of DMS being accompanied by formation of 2.2 mM sulfate. The low concentrations of dissolved DMS (Fig. 17) probably indicate that part of the added substrate was in the gas phase during growth, and dissolved during DMS consumption by the bacteria. This made dissolved-TOC measurements of little use except at the end of the growth phase. In a different experiment, growth of TK-m on a total of about 4 mM DMS (as three additions of about 1.4 mM, equivalent to 100 mg-carbon/liter) produced a final biomass of 49 mg-TOC/liter with 10 mg TOC remaining in solution. No methanol could be detected in the

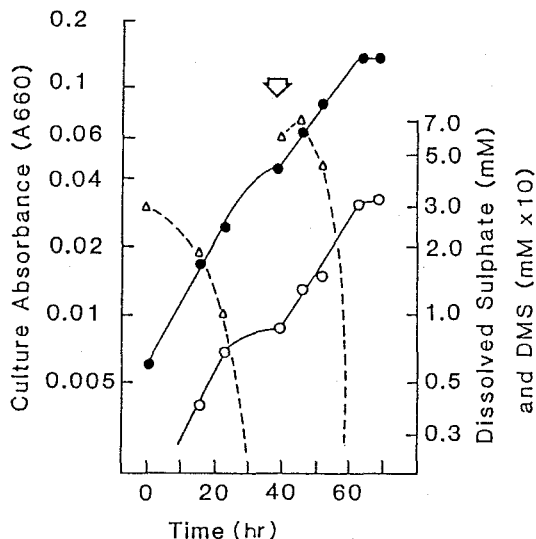


Fig. 17. Growth of Thiobacillus thioparus TK-m on DMS.

A batch culture (200 ml) was grown in a 500-ml sealed flask, shaken at 30°C, in a medium initially containing 1 mM DMS with a further 2 mM being added at 38 hr (arrow). More than half the added DMS can be seen to have been present in the gas phase in the flask immediately following addition of the 2 mM supplement.

●, A_{660} ; ○, sulfate; Δ, DMS dissolved in the medium.

cultures. It thus seemed that the thiobacillus degrade DMS to products other than solely sulfate and methanol. Separate experiments demonstrated that TK-m did not grow on, or degrade, methanol or dimethyl sulfate (DMSO_4), indeed DMSO_4 (1 mM) prevented growth of TK-m on DMS.

Pseudomonas sp. AK-2 did not grow on DMS when inoculated as a pure culture, but did sustain itself in mixed culture with TK-1 through several months of sequential subculture, indicating that a product of the metabolism of TK-1 was available to support AK-2. Strains AK-2 and AK-m grew readily on methanol and on DMSO_4

in the absence of strain TK-1.

Effect of pH and DMS concentration on the rate of growth of a mixed culture of strains TK-1 and AK-2

The mixed culture derived from the chemostat was inoculated into media of pH 5.6-7.7, containing 1.4 mM DMS. No growth occurred at pH 5.6 and the most rapid development was at pH 7.0-7.4 with rates of increase in A_{660} being halved at pH 6.1 and 7.7. Inoculation of the culture into medium at pH 7.0-7.2, containing 1.4, 2.8 or 5.6 mM DMS showed DMS to be an inhibitory substrate. Using 2.8 mM DMS the initial growth rate was less than a quarter of that on 1.4 mM, while 5.6 mM DMS caused a 3-day lag before growth at a comparably low initial rate commenced.

Source of cell-carbon for T.thioparus TK-m growing on DMS and $^{14}\text{CO}_2$

A culture of TK-m (100 ml) was grown on 2 mM DMS in the presence of 5 mM $\text{NaH}^{14}\text{CO}_3$ and the incorporation of ^{14}C correlated with the increase in biomass. After cessation of growth a further 3 mM DMS was added to allow growth and ^{14}C assimilation to continue (Table 3). Initially the cell-carbon was seen to be derived exclusively from $^{14}\text{CO}_2$, but the proportion of ^{14}C labeling relative to increase in biomass (as A_{660}) progressively declined. This indicated that DMS-carbon was not incorporated directly into the bacteria but appeared to depress ^{14}C fixation only after conversion to an assimilable carbon compound, presumably CO_2 . At the end of growth the culture had produced 66.5 mg of new cell TOC/liter, which was labelled at about 50% of the original bicarbonate-specific activity.

Table 3. Assimilation of $^{14}\text{CO}_2$ by Thiobacillus thioparus TK-m during growth on DMS.

A culture (100 ml in a sealed 500-ml flask) was grown initially on about 2 mM DMS and a further 3 mM DMS added at 48 hr. Culture absorbance at time 0 was 0.012; growth was complete by 100 hr when culture absorbance was 0.239. At that time the culture contained 103 mg-TOC/litre, of which 33 mg/liter was in the supernatant (including 16 ml/liter present as EDTA in the initial medium).

Time (hr)	Growth (as increase in absorbance at 660 nm) ^a	$^{14}\text{CO}_2$ fixed ^b (10^3 cpm)	Carbon incorporated by the bacteria(mg/liter)	
			$^{14}\text{CO}_2$ fixed ^b	Total cell carbon ^c
0	0	0	0	0
18	0.010	10.0	3.3	2.9
24	0.015	12.9	4.3	4.4
30	0.023	18.2	6.0	6.7
42	0.053	39.4	13.0	15.5
48	0.087	58.3	19.3	25.5
66	0.098	61.7	20.4	28.7
73	0.129	75.5	25.0	37.8
79	0.183	96.5	31.9	53.6
100	0.227	100.5	33.2	66.5

^a Given as A_{660} value after deduction of the initial value of 0.012.

^b Specific activity of the added $\text{NaH}^{14}\text{CO}_3$ was 18137 cpm/mol during the experiment.

^c Calculated from the relationship of A_{660} to cell TOC, in which an A_{660} value of 0.1 = 29.3 mg-TOC/liter.

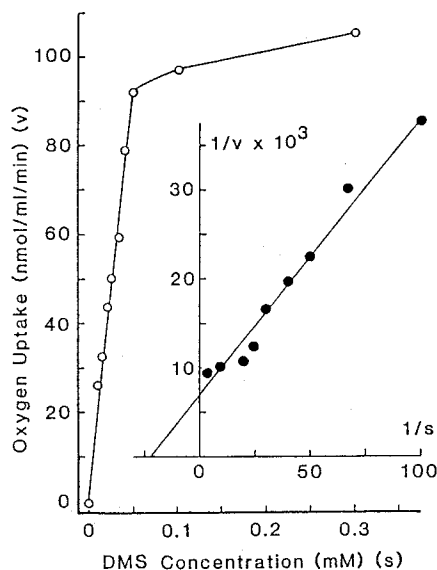


Fig. 18. Oxidation of DMS by Suspensions of Thiobacillus thioparus TK-m.

DMS was supplied at 0.01-0.3 mM to bacteria (0.3 mg-TOC/ml) in the oxygen electrode. Oxidation rates (nmol/ml·min) and their reciprocals (1/v) were plotted against DMS concentrations and their reciprocals (1/s). The abrupt transition from fast to slower increase in rates at high DMS concentrations possibly indicates DMS to be an inhibitory substrate, as does the V_{\max} of about 480 nmol- O_2 /mg-cell TOC·min which is 37% higher than the experimental maximum value (at 0.3 mM DMS) of 350 nmol/mg-TOC·min.

Oxidation of DMS by suspension of T.thioparus TK-m

Suspension of strain TK-m, previously grown on DMS, oxidized 0.01-0.3 mM DMS in the oxygen electrode cell (Fig. 18), exhibiting an apparent K_m of 4.5×10^{-5} M DMS. The oxidation rate of about 100 nmol O_2 /min·ml with 0.1-0.3 mM DMS was considerably greater than those of 11 and 8 nmol/min·ml for 0.17 mM thiosul-

fate and 0.25 mM DMDTP, respectively. The stoichiometry of oxygen uptake to DMS supplied was concentration-dependent in this environment, with an amount of oxygen consumed rising from about 0.9 mol-O₂/mol-DMS at 0.01 mM DMS, to about 2.1 mol/mol at 0.04-0.1 mM. Strain TK-m did not oxidize methanol, although this was rapidly oxidized by Pseudomonas sp. AK-m under the same conditions. DMS was not oxidized by strain AK-m.

Degradation of DMS by acclimated activated sludge

'Return sludge' from a sewage works (Minworth, Birmingham, U.K.) was diluted (1+2) with tap water, filtered through muslin and washed three times by decantation with tap water. Sludge (100 ml) was then allowed to settle for 30 min in a 500-ml bottle, 30 ml of the upper phase removed and replaced by 30 ml of a medium containing 0.2 g of KH₂PO₄, 0.2 g of K₂HPO₄, 0.1 g of MgSO₄·7H₂O, 0.1 g of NH₄Cl, and 0.1 g of yeast extract in 1 liter of tap water. This was shaken in air at 30°C for 24 hr, settled 30 min, and the top 30 ml replaced with 30 ml of the above medium containing 3.5 mM DMS. The bottle was sealed and shaken at 30°C for 2-3 days. This replacement procedure was repeated several times. After 22 days, a sample of the acclimated sludge (5 ml) was mixed with 45 ml medium B2 in a 300-ml flask, 7 mM DMS added and the sealed flask shaken at 30°C for 4 days. No DMS was detected and pH fell from 7.39 to 6.35. The enrichment was subcultured (5 ml) in the same way. After 4 days, pH fell to 6.56 and bacteria and flocks were observed. This culture was streaked onto agar plates of medium B2 and incubated in a sealed jar containing liquid DMS in equilibrium with the gas phase. No

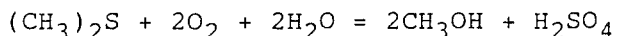
colony development was observed after 7 days. Further subculture of the liquid enrichment showed poor growth, even when the medium was supplemented with vitamins. The acclimated sludge continued to degrade DMS over a long period but no pure culture of a DMS-degrading organism could be obtained on solid media, although it was possible to obtain colonies of Thiobacillus-like bacteria on thiosulfate agar.

DISCUSSION

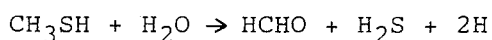
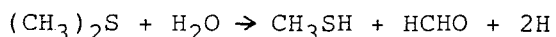
A Thiobacillus-like organism able to degrade DMS was previously isolated from a cellulose mill (4) but author's present report is the first demonstration that a pure culture of T.thioparus, isolated originally from activated sludge, is capable of growing on DMS and producing sulfate quantitatively from it. The implication of this finding, and the observations on acclimated sludge, is that the degradation of methyl sulfides in sewage treatment and, more significantly, in natural environments, may be catalyzed by thiobacilli. This work also indicates that some degree of commensalism could occur, involving mixtures of thiobacilli and methylotrophs.

The most interesting biochemical observation emerging from this study is that T.thioparus TK-m clearly grows autotrophically while oxidizing DMS and does not appear to assimilate DMS-carbon by 'methylotrophic' mechanisms such as the serine pathway. The mechanism of DMS breakdown is, however unclear. Strain TK-m does not appear to produce methanol during growth, although the

oxidation stoichiometry in the oxygen electrode was consistent with the following reaction:

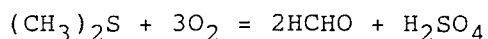


Growth in the presence of $^{14}\text{CO}_2$ and DMS showed that a product of DMS oxidation diluted the specific activity of the newly synthesized cell-carbon in such a way as to indicate that DMS was converted to CO_2 , or to a compound competing with CO_2 for assimilation by the bacteria. The observed growth yields of 12-13 g-cell TOC/mol-DMS consumed is consistent with that expected for an autotrophic bacterium rather than a serine pathway or Quayle cycle methylotroph (30). Observed yields of thiobacilli growing on sulfide or thiosulfate are in the range 2-5 g-TOC/mol (31,32) and for T.versutus growing autotrophically on methanol the yield is 2-3 g-TOC/mol (33,34). The author can tentatively conclude that T.thioparus TK-m exhibits the observed growth yield by obtaining energy from the equivalent of oxidizing one 'sulfide' and two 'methanol' molecules if it converts DMS completely to sulfate and CO_2 . As yet the author has no enzymological data on the nature of the initial attack on DMS. By analogy with the metabolism of dimethylamine and methylamine (35), the initial product of DMS oxidation could be formaldehyde without the intermediate production of methanol, thus explaining our failure to detect methanol formation by whole cells:



The H_2S (if actually liberated as a free intermediate) and the reducing equivalents would be oxidized via the electron transport

chain by oxygen, giving an overall stoichiometry of:



Formaldehyde would presumably undergo further oxidation to CO_2 . It is apparent that this sequence of reactions is of considerable interest since the organism, T.thioparus TK-m, is regarded as an obligate autotroph, normally obtaining energy only from inorganic sulfur oxidation.

SUMMARY

DMS was degraded by acclimated activated sludge and by a mixed culture of Thiobacillus thioparus TK-1 and Pseudomonas sp. AK-2. While both these organisms persisted in stable co-culture on DMS, it was found that T.thioparus TK-1 and the derived strain TK-m grew in pure culture on DMS, and oxidized DMS with an apparent K_m of 4.5×10^{-5} M. During growth, all the DMS-sulfur was oxidized stoichiometrically to sulfate but no methanol was detected in pure cultures of TK-m. DMS-carbon was probably converted to CO_2 , since the fixation of $^{14}\text{CO}_2$ was progressively diluted during growth of a culture on $^{14}\text{CO}_2$ and DMS. Growth yields were consistent with autotrophic growth, dependent on the oxidation of the methyl residues to CO_2 (probably with formaldehyde as a first intermediate) and the sulfide to sulfate. The organism thus appears to exhibit a mixture, from the one substrate, of chemolithotrophic and methylotrophic energy generation supporting autotrophic growth with CO_2 fixation.

CHAPTER 4 Removal of Methyl Mercaptan, Dimethyl Sulfide,
Dimethyl Disulfide, and Hydrogen Sulfide from
Contaminated Air by Thiobacillus thioparus TK-m^d)

INTRODUCTION

In order to develop efficient methods for biological deodorization, the deodorizing potential of Thiobacillus thioparus TK-m was examined. Results of this study indicate that MM, DMS, DMDS, and H₂S can be efficiently removed from contaminated air by use of this strain.

MATERIALS AND METHODS

Organism, media and preculture conditions. The isolation of T.thioparus TK-m has been described in Chapter 3. Basal medium C described in Chapter 3 was used in precultures and deodorization tests. A preparation of medium CS (2.5 g of Na₂S₂O₃·5H₂O and 15 g of agar added to 1 liter of medium C) was used to maintain T.thioparus TK-m and to measure the number of T.thioparus TK-m cells in the cultures. Medium N, which contained 2 g of nutrient broth (Difco Laboratories, Detroit, Mich., U.S.A.) and 15 g of agar in 1 liter of distilled water, was used to measure the number of heterotrophic cells in the cultures. Precultures (50 ml) were grown on DMS in 300-ml flasks that were sealed with rubber stoppers, as described in Chapter 3, and shaken at 25°C (unless indicated otherwise).

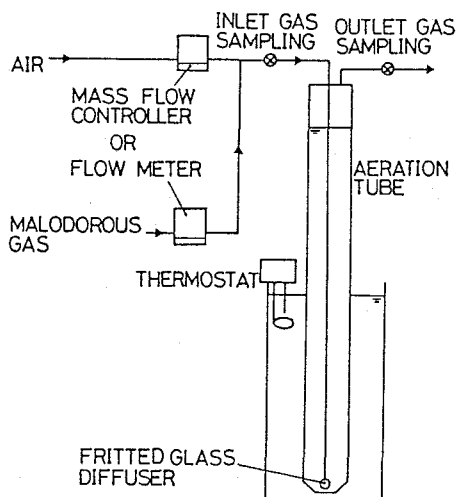


Fig. 19. A Schematic Diagram of a Deodorizing Apparatus Used in This Study.

Deodorizing tests. A schematic diagram of the deodorizing apparatus used in this study is shown (Fig. 19). The acrylic tubes (55 mm by 1 m) were filled with 700 ml of precultures made up of *T.thioparus* TK-m and 1300 ml of sterilized medium C. Malodorous gases (1-5 ml/liter of nitrogen) were diluted with air and supplied to the bottom of the tubes through fritted glass diffusers. The flow rate of the gases and the air was controlled by mass flow controllers (Teledyne Hastings-Raydist, U.S.A.) or was regulated and monitored by flow meters. The bacterial cultures in the tubes were maintained at 25°C and at pH 6.8 ± 0.4 by titration with 1 M K₂CO₃.

Analytical methods. Growth of the cultures in deodorizing tubes was monitored by either measuring the A₆₆₀ in a spectrophotometer (101; Hitachi), or measuring the TOC in the cells in a TOC analyzer (TOC-500; Shimadzu). TOC in cells represented TOC in cultures after the deduction of TOC from

culture filtered through membrane filters (pore size, 0.45 μm ; Millipore Corp., Bedford, Mass., U.S.A.). Dry cell weights were calculated from the relationship to A_{660} , in which an A_{660} value of 0.1 was equal to 75.5 mg-dry cell/liter. Cell yields were calculated on the basis of increased A_{660} values and increased sulfate concentrations. In order to measure the number of cells, 1 ml of culture was diluted with sterilized water and 0.1 ml of the diluted liquid was applied to a medium in a plate. This medium in the plate was incubated for between 9 and 11 days at 25°C and the number of visible colonies was counted. The number of round colonies with precipitated elemental sulfur appearing on medium CS were determined as the number of T.thioparus; the number of colonies on medium N was determined as the number of heterotrophs. Sulfate was determined with an ion chromatographic analyzer (IC500; Yokogawa Hokushin Electric, Tokyo, Japan). MM, DMS, DMDS and H_2S were measured by a gas chromatograph (GC-5A; Shimadzu) equipped with a flame photometric detector and a glass column (length, 3 m) packed with 25% β,β' -oxydipropionitrile on 60-80 mesh Chromosorb W. The lower limit for detection was 0.1 μl /liter. Human volunteers were used to detect malodorous compounds and proved to be much more effective than the gas chromatographic technique. It has been reported that human volunteers recognize 2.1 nl of MM/liter, 1.0 nl of DMS/liter and 0.47 nl of H_2S /liter (1).

Gases. MM, DMS, DMDS and H_2S gases (1-5 ml/liter of nitrogen) in 10-liter or 40-liter gas tanks were obtained from Seitetsu Chemical Industries Ltd. (Osaka, Japan) and Takachiho

RESULTS

Removal of malodorous compounds by a sterilized medium

The aeration tube was filled with 2 liters of sterilized medium C. DMS gas (60 $\mu\text{l/liter}$) was supplied at 250 $\text{Ncm}^3(\text{Ncm}^3, \text{volume when the gas is at } 0^\circ\text{C and } 1 \text{ atm})/\text{min}$. The DMS concentration of outlet gas after 5 hr was as high as that of the inlet gas. There was no increase of sulfate concentration in the medium over a 24-hr period. Thus, DMS was not removed by the sterilized medium. MM gas and DMDS gas showed the same behavior pattern in the sterilized medium.

H_2S gas (140 $\mu\text{l/liter}$) showed a different behavior pattern (Fig. 20). The H_2S concentration of outlet gas after 24 hr was only 20 $\mu\text{l/liter}$. The absorbance of the medium increased,

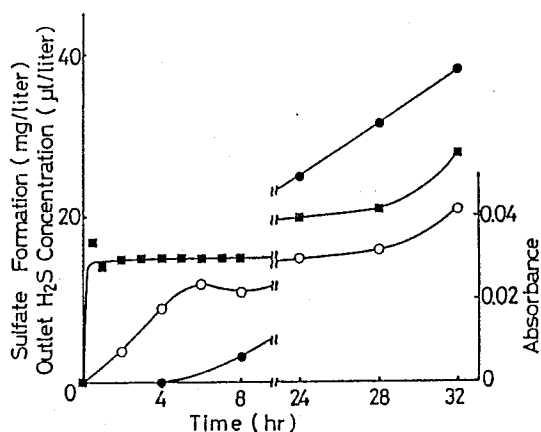


Fig. 20. Removal of H_2S by a Sterilized Medium. H_2S gas (140 $\mu\text{l/liter}$) was supplied at 250 Ncm^3/min . ■, outlet H_2S concentration; O, A_{660} ; ●, sulfate.

probably because of the reaction between H_2S and the metal ion in the medium, to form insoluble sulfides. Yellowish white precipitates, that were thought to be elemental sulfur appeared in the bottom of the tube. No sulfate was formed during the first 4 hr of gassing in the reactor. However, 3.2 mg of sulfate/liter formed after 8 hr and 25.0 mg/liter after 24 hr, indicating that 9 % and 24 % of supplied H_2S were oxidized to sulfate, respectively. Thus, a fairly large amount of H_2S was removed by the sterilized medium, but complete removal was impossible.

Removal of DMS by *T.thioparus* TK-m

DMS gas (100 μ l/liter) was supplied at 250 Ncm³/min to the aeration tube that was inoculated with *T.thioparus* TK-m (Fig. 21). Gas bubbles reached the surface within 3 seconds. The initial cell concentration was an A_{660} value of 0.008. The outlet gas contained 25 μ l/liter of DMS after 2 hr, but DMS was not detected after 24 hr (A_{660} , 0.032) or subsequently in outlet gas by either gas chromatography or the human volunteers. The culture was uniformly turbid at all times. No bacterial flocks appeared in the culture. Almost all the cells that were observed through a microscope were small bacilli. Plate counts showed relative numbers of the organisms in the 10-day culture to be 10 TK-m to 1 heterotroph. Cell yield in the initial 10 days was 21 g-dry cell/mol-DMS. Sulfate was formed stoichiometrically. Growth of the cells terminated when the A_{660} value reached nearly 1.0, probably because of the lack of a nitrogen source.

Next, the maximum degradation rate of DMS by the cells was

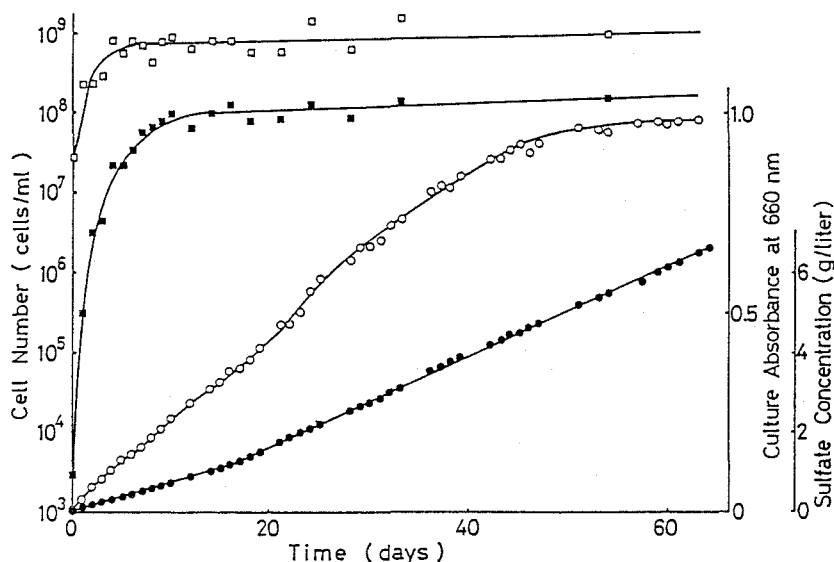


Fig. 21. Removal of DMS by T.thioparus TK-m. DMS gas (100 μ l/liter) was supplied at 250 Ncm³/min. After 16 days, the DMS concentration of the inlet gas was increased to 150 μ l/liter. The outlet gas contained 25 μ l/liter of DMS after 2 hr, but DMS was not detected after 1 day or subsequently in the outlet gas by either gas chromatography or the human volunteers.

O, A₆₆₀; ●, sulfate; ■, cell number of T.thioparus TK-m; □, number of heterotrophic cells.

measured. A total of 20 ml of a 40-day culture was transferred to another aeration tube containing 1980 ml of medium C. DMS gas (50 μ l/liter) was supplied for 4 days at 250 Ncm³/min; then, the DMS concentration was increased stepwise (Fig. 22). When the DMS load was not over 4.0 g/g-dry cell·day, no DMS was detected in the outlet gas by either gas chromatography or the human volunteers. This shows that the maximum degradation rate was 4.0 g/g·day.

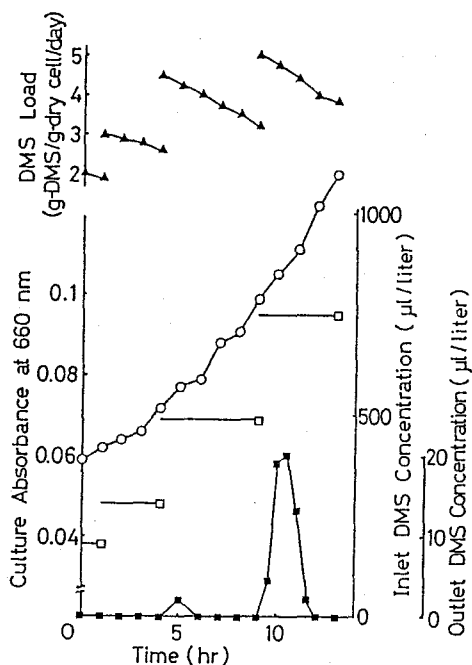


Fig. 22. Removal Efficiency of DMS for the Different Loadings.

DMS gas (180-750 $\mu\text{l/liter}$) was supplied at 250 Ncm^3/min .

O, A_{660} ; □, inlet DMS concentration; ■, outlet DMS concentration; ▲, DMS load.

In order to estimate the maximum capacity of the apparatus to remove DMS, the DMS load to the aeration tube was increased stepwise according to cell growth. A medium that was prepared by adding 1.8 g of NH_4Cl and 4 ml of trace metal solution to 1 liter of medium C was used in this experiment. DMS gas (100-760 μl) was supplied at 500 to 1610 Ncm^3/min . The maximum DMS load in this experiment was 2.42 g/liter·day. When the load reached maximum, the cell concentration was 1.02 g/liter, and DMS gas (760 $\mu\text{l/liter}$) was supplied at 1610 Ncm^3/min . More DMS did not

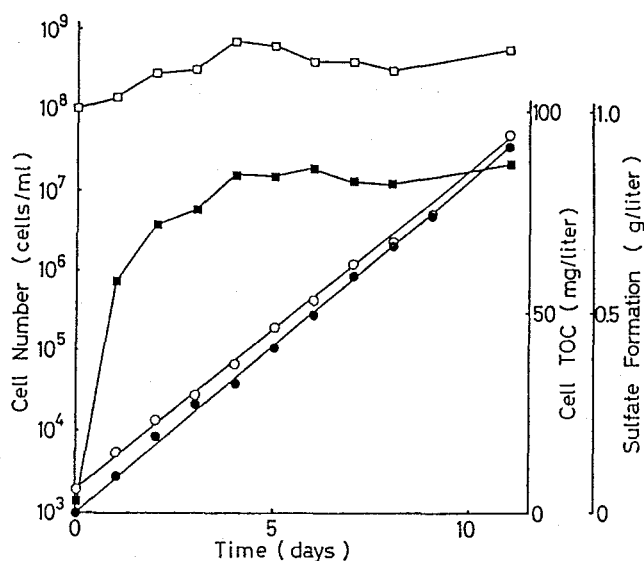


Fig. 23. Removal of MM by T.thioparus TK-m.

MM gas (106 $\mu\text{l/liter}$) was supplied at 250 Ncm^3/min . MM was not detected in outlet gas at any time throughout the experiment by either gas chromatography or the human volunteers.

O, cell TOC; ●, sulfate; ■, cell number of T.thioparus TK-m; □, number of heterotrophic cells.

flow into the aeration tube, probably because of the narrow pipe lines. DMS was not detected in the outlet gas in this experiment.

Removal of MM by T.thioparus TK-m

MM gas (106 $\mu\text{l/liter}$) was supplied to the aeration tube at 250 Ncm^3/min (Fig. 23). The initial cell concentration was an A_{660} value of 0.028. The initial MM load to the cells was 2.0 g/g-dry cell·day. MM was not detected in the outlet gas at any time throughout the experiment by either gas chromatography or the human volunteers. Sulfate was formed stoichiometrically. After 11 days, plate counts showed the relative number of

organisms to be 25 TK-m to 1 heterotroph. The cell yield on MM was 20 g/mol-MM.

Removal of H_2S by *T.thioparus* TK-m

H_2S gas (220 μ l/liter) was supplied at 250 Ncm³/min. The initial cell concentration was an A_{660} value of 0.028. The initial H_2S load to the cells was 2.9 g/g-dry cell·day. H_2S was not detected in outlet gas at any time. Sulfate was formed stoichiometrically. As described above, H_2S oxidation occurred in sterilized medium C, but the rate was very low. Therefore, in this experiment, H_2S oxidation was thought to be carried out mainly by the microorganisms. After 11 days, the relative number of organisms was 10 TK-m to 1 heterotroph. The cell yield on H_2S was 9.2 g/mol- H_2S .

Removal of DMDS by *T.thioparus* TK-m

Precultures grown on DMDS were inoculated into the aeration tube. The initial cell concentration was an A_{660} value of 0.018. DMDS gas (65 μ l/liter) was supplied at 250 Ncm³/min. The initial DMDS load to the cells was 3.6 g/g-dry cell·day. Outlet gas contained 3 μ l/liter of DMDS after 2 hr and 5 hr, but no DMDS was detected in the outlet gas after 24 hr (A_{660} , 0.042). Sulfate was formed stoichiometrically. The cell yield on DMDS was 33 g/mol-DMDS. Precultures grown on DMS did not degrade DMDS in the aeration tube over 3 days.

DISCUSSION

This is the first study in which it has been demonstrated

that methyl sulfides are removed from contaminated air by an identified microorganism and are oxidized to sulfate stoichiometrically. More than 99.99% of the DMS was removed by T.thioparus TK-m, when the DMS load to the cells was 4.0 g/g-dry cell·day. Fukuyama et al. (36) have reported that the maximum DMS load for acclimated activated sludge to obtain a 99.9% removal was 9.0 mg/g-MLSS·day. The maximum load for removing more than 90% of reduced sulfur contained in kraft pulp wastewater, i.e. MM, DMS, DMDS and H₂S, by activated sludge has been reported to be 15 mg-S/g-MLSS·day (37). Therefore, the cultures of T.thioparus TK-m removed DMS 100-fold more rapidly than the acclimated activated sludges. The cultures in the aeration tube degraded DMS completely for more than two months. Moreover, MM, DMDS and H₂S were also removed by T.thioparus TK-m as efficiently as DMS was. Thus, T.thioparus TK-m proved very useful for removing malodorous methyl sulfides and hydrogen sulfide.

After the inoculation of pure cultures of T.thioparus TK-m in the aeration tube, heterotrophs grew very rapidly. But the plate counts always showed that the relative number of organisms was more than 10 TK-m to 1 heterotroph. As described in Chapter 3, T.thioparus TK-m utilizes not only reduced sulfur, but also the carbons in the DMS molecule; consequently, there was very little of the energy source remaining in the culture broth after the growth of T.thioparus TK-m. Thus, the mixed cultures in the aeration tube consisted of a large amount of strain TK-m but little of the other organisms.

The cell yields on H₂S and DMS were 9.2 and 21 g/mol,

respectively. These values are very similar to those for Hyphomicrobium sp. EG, the yields of which were reported to be 8 to 10 and 19.1 g/mol of consumed H_2S and DMS, respectively (38). The cell yields on MM and DMDS were 20 and 33 g/mol, respectively. It was very interesting to observe from the point of view of a biochemist that there was very little difference between the cell yield on MM and that on DMS.

SUMMARY

MM, DMS, DMDS, and H_2S were efficiently removed from contaminated air by Thiobacillus thioparus TK-m and oxidized to sulfate stoichiometrically. More than 99.99% of DMS was removed when the load was less than 4.0 g-DMS/g-dry cell·day.

CHAPTER 5 Removal of Methyl Mercaptan, Dimethyl Sulfide, and
Hydrogen Sulfide by Immobilized Thiobacillus
thioparus TK-m^{e)}

INTRODUCTION

Thiobacillus thioparus TK-m showed excellent decomposing ability for MM, DMS, DMDS, and H₂S as described in Chapter 4. By the effective use of this bacterium, equipment capable of efficiently treating sulfur-containing malodorous gas is expected to be developed. However, there have been no examples of application of identified bacteria for the treatment of malodorous gas.

In designing an apparatus to treat a large quantity of malodorous gas at a low concentration, a reduction in pressure loss in the apparatus is important to economize the operating cost. The author marked the packed tower system that features smaller pressure loss and easier maintenance, and constructed an experimental apparatus having a cylinder packed with plastic pellets that immobilized T.thioparus TK-m. The efficient removal of DMS, MM, and H₂S by this apparatus is described in this chapter.

MATERIALS AND METHODS

Organism and medium. The isolation of T.thioparus TK-m has been described in Chapter 3. This strain was maintained on

agar slants containing 10 mM of $\text{Na}_2\text{S}_2\text{O}_3$.

Basal medium D consisted of 2 g of K_2HPO_4 , 2 g of KH_2PO_4 , 0.4 g of Na_2CO_3 , 3.8 g of KNO_3 , 0.2 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5 ml of trace metal solution (28) and 5 ml of vitamin mixture (described in Chapter 2) in 1 liter of distilled water.

Preculture and immobilization. Strain TK-m was precultured on DMS in flasks, mixed with activated sludge, transferred to an aeration tank, cultured on DMS again, and then adhered to polypropylene pellets. The activated sludge was used as a glue to adhere strain TK-m to the pellets. The procedure is described in detail below.

Each of four 500-ml erlenmeyer flasks was charged with 150 ml of medium D and 3.4 mg of DMS, inoculated with strain TK-m, sealed with a rubber stopper, and shaken for two weeks at 25°C . DMS was added according to the growth of the bacteria. The total amount of DMS added was 35-40 mg per flask.

Medium D (1100 ml) was added to the obtained culture (600 ml), which was then mixed with activated sludge (300 ml) that had been filtered through muslin and washed twice by decantation with medium D. This mixture (2 liters in total) was introduced into an acrylic aeration tank (55 mm inner diameter, 1000 mm long), to which DMS gas at a concentration of 20 $\mu\text{l/liter}$ was supplied at a flow rate of 500 ml/min. The concentration of DMS gas was increased according to the growth of bacteria and maintained at 70 $\mu\text{l/liter}$ on and after the 12th day. The cultural pH was maintained in the range of 6.5-7.0 by addition of 2 M KHCO_3 . After 20 days, the culture was allowed to stand until the cells

settled. One liter of the supernatant was then discarded and the 1 liter suspension (cell weight 500 mg) was obtained.

Into this suspension, 2 liters of cylindrical porous polypropylene pellets (5 mm ϕ x 5 mm, apparent density 0.3, water retention 215 g/liter) were introduced, and the mixture was degassed under a vacuum with agitation to impregnate the cells into the pores of the pellets. The pellets were packed in an acrylic cylinder (100 mm inner diameter, 1200 mm long, packed height 250 mm), and DMS gas (4 μ l/liter) was supplied to the packed bed at a flow rate of 500 ml/min. Medium D was intermittently supplied to the packed bed. Supply of DMS was gradually increased and, on and after the 22nd day, a DMS concentration of 20 μ l/liter and a flow rate of 8 liter/min were maintained. The culture was continued for 66 day. Cell concentrations immobilized on the pellets after the culture (g-dry cells/liter) were: 1.5 in the upper part of the packed bed, 4.4 in the middle part, and 5.2 in the lower part. The whole pellets were taken out of the cylinder and charged into the experimental packed tower apparatus, as described below, after sufficient mixing of the pellets that came from different parts of the cylinder.

Experimental packed tower apparatus and experimental conditions. The experimental packed tower apparatus is shown in Fig. 24. The packed tower consisted of an acrylic cylinder (50 mm inner diameter, 1250 mm long) and pellets with immobilized cells packed to the height of 800 mm (pellets volume 1.6 liter). Five sampling ports were provided at 200-mm height intervals

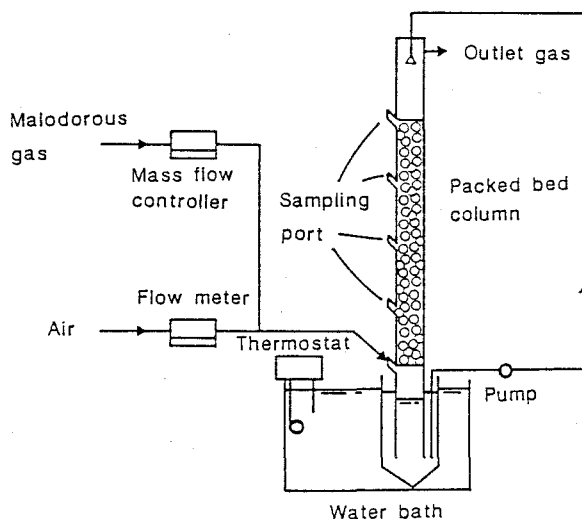


Fig. 24. Experimental Packed Tower Apparatus.

along the packed bed. Malodorous gas was supplied under flow control by a mass flow controller (Teledyne Hastings-Raydist, Hampton, U.S.A.), and introduced to the lower part of the packed tower after dilution with air to a prescribed concentration. DMS gas (30 $\mu\text{l/liter}$) was supplied at a flow rate of 6 liter/min, unless otherwise described. The lower end of the packed tower was water-sealed by positioning an acrylic container (100 mm inner diameter, 300 mm high) containing 2 liter of medium D. Unless otherwise described, this liquid was circulated to the upper part of the tower at a flow rate of 200 ml/min for 10 seconds at 10 min intervals. The pH of the circulating liquid was maintained in the range of 6.5 - 7.0 by the addition of 2 M KHCO_3 . When the concentration of sulfate ion, a decomposition product from sulfur-containing malodorous gas, in the circulating liquid exceeded 5 g/liter, 3/4 of the liquid was replaced by fresh medium. The loss of water caused by aeration was compen-

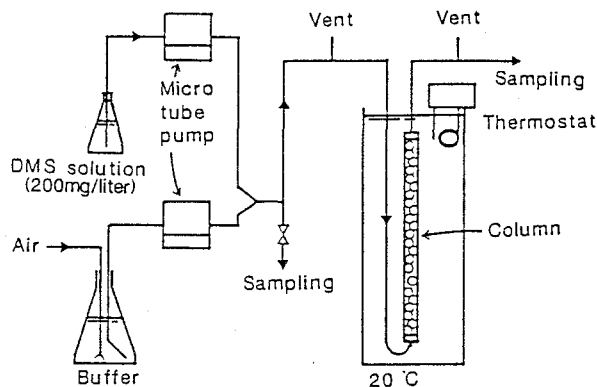


Fig. 25. Experimental Apparatus for Decomposition of Dissolved DMS.

sated for by the addition of distilled water. The temperature of the circulating liquid was maintained at 25°C.

Experiments on the decomposition of malodorous gas were carried out by the following procedure. Firstly, the liquid was circulated for about 5 min at a flow rate of 1.6 liter/min to thoroughly wash the pellets. The liquid circulation was then stopped. Next, gas with a prescribed concentration was supplied at a prescribed flow rate, and the gas concentration at the outlet was determined at 10 min and 20 min after stopping circulation of the liquid. Since the analyzed values for 10 min and 20 min were little different, the average of both values was taken as the gas concentration at the outlet. The experiments were carried out at room temperature.

Experimental apparatus for the decomposition of dissolved DMS and experimental conditions. The experimental apparatus for the decomposition of dissolved DMS is shown in Fig. 25. Pellets removed from the experimental packed tower were packed

into an acrylic cylinder (20 mm inner diameter, 480 mm long) to which phosphate buffer (KH_2PO_4 1 g/liter, K_2HPO_4 1 g/liter, Na_2CO_3 0.2 g/liter, pH 7) was supplied at a flow rate of 10 ml/min for about 15 hr. After confirmation that the sulfate ion concentration of the column effluent was within the detection limit (40 $\mu\text{g/liter}$) of the ion-chromatography system, a mixture of aqueous DMS solution (200 mg/liter) and phosphate buffer was supplied to the column at a constant flow rate. The decomposition rate was evaluated from the determination of sulfate ion (the decomposition product from DMS) concentration of the column effluent. Unless otherwise described, experiments were carried out under the following conditions: flow rate of the mixed solution: 22 ml/min, concentration of DMS: 10 mg/liter, and temperature: 20°C.

Analysis. The cell concentrations immobilized on pellets were calculated from the nitrogen content of the pellets determined by neutralization titration after pretreatment of the pellets by Kjeldahl's method. Analyses of DMS, MM, and H_2S were made with gas chromatography as described in Chapter 4; analysis of sulfate ion was carried out with an ion chromatographic analyzer IC500 (Yokogawa-Hokushin Electric); and analysis of ammonium ion was conducted using a gas indicator tube No.3L (Gastec Corp.). The ammonium ion concentration of the circulating liquid was analyzed by neutralization titration of a steam-distilled sample.

Gases. MM gas (5 ml/liter of nitrogen), DMS gas (2 ml/liter of nitrogen), H_2S gas (20 ml/liter of nitrogen) and

ammonia gas (20 ml/liter of nitrogen) in 10-liter or 40-liter gas tanks were obtained from Seitetsu Chemical Industries Ltd. (Osaka, Japan).

RESULTS

Pressure loss in the packed tower

Pressure loss was measured immediately after packing the pellets in the packed tower. Measurements were made using manometers attached to the sampling ports 5 min after the liquid circulation was stopped. The temperature of the air supplied was 22°C. The pressure loss in the tower (g/cm^2) was proportional to the height of the packed bed, and was 1.13, 3.8, 8.1, and 13.5 per 1000 mm of the packed bed height for air flow rates (liter/min) of 12, 24, 36, and 47, respectively.

Decomposition of DMS, MM and H_2S

For evaluation of the decomposition capability of the experimental packed tower for malodorous gas, the influence of the liquid circulation on decomposition was first examined. While DMS gas (3.5 $\mu\text{l/liter}$) was supplied to the packed bed at a flow rate of 12 liter/min, liquid circulation at a flow rate of 100 ml/min was continued for 1 hr and then stopped. During liquid circulation, the outlet DMS concentration was about 0.6 $\mu\text{l/liter}$, and the removal rate was about 80%; however, 5 min after the termination of circulation, the outlet DMS concentration was 0.14 $\mu\text{l/liter}$, and the removal rate was about 96%. The same values were observed even after 10 and 30 min. A

removal rate over 96% was maintained for an additional 4 hr; however, 4.5 hr after the termination of liquid circulation, the outlet DMS concentration rose sharply to 0.82 $\mu\text{l/liter}$. By restarting the circulation, the outlet DMS concentration nearly recovered to the same level as that observed during the initial circulation.

Based on this experimental result, in order to maintain a constant contribution by the liquid circulation on the decomposition data, the outlet gas concentration was determined at 10 and 20 min after the stoppage of liquid circulation as described in the Materials and Methods.

After packing the pellets, decomposition experiments were carried out on the 9th day for DMS, on the 26th day for MM, and on the 28th day for H_2S (Table 4). The removal rates were measured by stepwise increases in the inlet malodorous gas concentration. When a malodorous gas was supplied at a flow rate of 12 liter/min (superficial velocity 0.1 m/s), the maximum loading rates (mmol/liter \cdot day) to attain a removal rate of 95% or more were 3.65, 8.74, and 17.36 for DMS, MM, and H_2S , respectively. In the case of a flow rate of 36 liter/min (superficial velocity 0.3 m/s), the percent removal decreased greatly. Because the cell concentrations on the pellets were not the same for different malodorous compounds in the decomposition experiments, the relative ease of decomposition for each compound cannot be discussed by comparing experimental values. For every compound, a higher loading rate resulted in greater removal quantities, and this tendency was more conspicuous for cases with

Table 4. Decomposition of DMS, MM and H₂S

Malodorous compound	Flow rate (liter /min)	Gas concentration at 200 mm intervals from the inlet (μ l/liter)					Loading rate (mmol /l·d) ^a	Removal rate	
		0mm (inlet)	200mm	400mm	600mm	800mm (outlet)		(mmol /l·d) ^a	(%)
DMS ^b	12	1.79	1.00	0.37	0.11	<0.05	0.88	>0.85	>97
		3.48	2.33	1.05	0.36	0.10	1.71	1.66	97
		7.44	5.52	2.46	1.12	0.28	3.65	3.52	96
		14.49	12.52	7.73	5.87	3.27	7.11	5.51	77
		20.66	17.16	14.10	12.20	8.44	10.14	6.00	59
	36	1.91	1.66	1.32	0.96	0.66	2.81	1.84	65
		3.86	3.50	3.07	2.68	2.14	5.69	2.53	45
		7.39	6.75	6.26	5.29	4.81	10.89	3.80	35
		12.07	11.47	10.85	10.18	8.86	17.78	4.73	27
		16.64	15.72	14.82	13.96	13.11	24.51	5.20	21
MM ^b	12	3.35	1.91	0.53	0.12	<0.05	1.64	>1.62	>99
		7.73	5.54	2.46	0.59	0.16	3.80	3.72	98
		17.80	13.17	5.60	2.87	0.76	8.74	8.37	96
		33.53	29.75	17.29	10.23	4.54	16.46	14.23	87
	36	4.49	4.01	3.35	2.02	1.35	6.61	4.63	70
		7.02	5.80	4.74	4.24	2.80	10.34	6.22	60
		12.59	11.27	9.15	8.29	6.35	18.55	9.19	50
		18.94	16.94	15.66	13.39	10.94	27.90	11.78	42
		40.03	37.24	31.31	28.09	25.41	58.96	21.54	37
		6.79	3.37	1.64	0.73	0.14	3.33	3.27	98
H ₂ S ^b	12	17.14	13.14	4.59	2.02	0.43	8.42	8.20	98
		35.36	22.03	13.14	5.92	1.62	17.36	16.57	95
		59.86	35.62	26.45	14.81	5.51	29.39	26.69	91
		8.50	7.24	6.06	4.77	3.24	12.52	7.75	62
	36	19.08	17.01	14.49	12.22	8.91	28.10	14.98	53
		36.91	32.90	28.98	23.26	18.83	54.37	26.63	49
		64.66	59.23	49.88	43.20	37.41	95.24	40.14	42

^a mmol/liter·day.^b The temperature of the outlet gas was : 23°C for DMS, 21°C for MM, 21°C for H₂S.

the lower flow rate. Immediately after the termination of the experiment with H_2S (on the 28th day after packing the pellets), cell concentrations immobilized on the pellets (g-dry cells/liter of pellets) were measured. The values obtained were 3.6, 4.6, and 11.7 for the upper, middle, and lower parts of the packed bed, respectively. This result indicates that the lower part with the higher substrate concentration sustained over three times as much of the immobilized cells compared to the upper part. However, as indicated in Table 4 for the gas concentration values at 200-mm intervals from the inlet, the decomposition capabilities for malodorous compounds were essentially the same for the upper and lower parts, and had little to do with immobilized cell concentration. As for the decomposition of DMS, a similar experiment was performed on the 62nd day, and it was found that the decomposition capability was nearly the same as that in the experiment on the 9th day, regardless of the much larger immobilized cell concentration.

By inspection with an optical microscope, microorganisms other than bacteria, such as Rotatoria and Nematoda, as Metazoa, and Mastigophora and Ciliata, as Protozoa, were observed.

Decomposition of DMS and H_2S mixed gas

Decomposition experiments on a gas containing both DMS and H_2S were carried out (Fig. 26). The coexistence of H_2S (60 μ l/liter) decreased the removal rate of DMS slightly. The decrease was only 1.3% for an inlet DMS concentration of 4 μ l/liter, and 3.5% for 15 μ l/liter. On the other hand, the removal rates of H_2S were 96.1% and 92.7% for DMS coexistence at

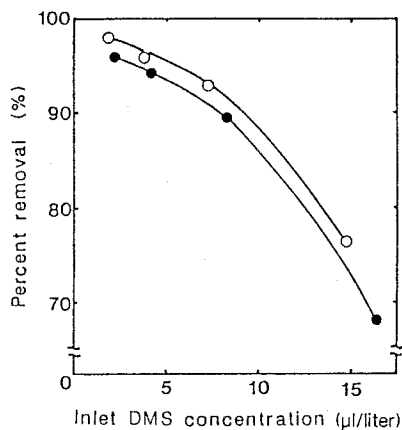


Fig. 26. Decomposition of the Mixed Gas Consisting of DMS and H₂S.

First, the removal rates of DMS (O) were determined with the supply of 2-15 μl/liter of DMS only at a flow rate of 12 liter/min. Second, removal rates of DMS (●) were obtained with the supply of DMS (2-16 μl/liter) and H₂S (60 μl/liter).

concentrations of 2 μl/liter and 16 μl/liter, respectively. The difference was only 3.4%. In this experiment, DMS removal of 96% was attained in the run with an inlet DMS concentration of 4 μl/liter and the absence of H₂S, while H₂S removal of 96% was attained in the run even with an inlet concentration of 60 μl/liter; therefore, easier decomposition of H₂S than DMS was verified. It was found that the coexistence of easily decomposable H₂S hardly affected the decomposition of DMS.

Influence of ammonia

Ammonia coexists with sulfur-containing compounds in malodorous gas in night soil and sewage treatment facilities. Therefore, its influence on strain TK-m was examined. First, a mixed gas of ammonia and DMS (NH₃ 26 μl/liter, DMS 1.6 μl/liter)

was supplied to the packed tower at a flow rate of 12 liter/min, and the DMS concentration of charged gas was increased stepwise up to 9.0 $\mu\text{l/liter}$. Next, a similar experiment was conducted without the addition of ammonia and the results of both experiments were compared. There was no difference at all in the outlet DMS concentration between the case with the presence and the case with the absence of ammonia at the inlet, indicating that ammonia had no influence on the decomposition of DMS. The ammonia concentration in the outlet gas was always 0.5 $\mu\text{l/liter}$, having no relation to the variations in DMS concentration of the inlet gas. An increase in ammonium ion equivalent to the ammonia supplied was recognized when a mixed gas of ammonia and DMS (NH_3 18 $\mu\text{l/liter}$, DMS 30 $\mu\text{l/liter}$) was supplied to the packed tower at a flow rate of 6 liter/min for 4 days. In this experiment, ammonia was not detected in the outlet gas; this is evidence that the ammonia supplied entered the liquid totally in the form of ammonium ions.

Decomposition of dissolved DMS

Using the experimental apparatus for decomposition of dissolved DMS, the rate of DMS decomposition by immobilized bacteria was examined. First, a phosphate buffer solution and an aqueous DMS solution were supplied to the apparatus at flow rates of 54 ml/min and 0.7 ml/min, respectively. The flow rate of DMS solution (ml/min) was then raised stepwise to 1.3, 2.1 and 3.4 at 2-hr intervals. The decomposition rate of DMS per unit volume of pellets was 7.05 mmol/liter \cdot day at the maximum when DMS solution was supplied at a flow rate of 2.1 ml/min (inlet DMS

concentration was 7.5 mg/liter). When the flow rate of the DMS solution was raised to 3.4 ml/min (inlet DMS concentration was 11.8 mg/liter), the decomposition rate of DMS decreased a little. The immobilized cell concentration in this experiment was 6.5 g/liter, so that the maximum DMS decomposition per unit cell weight was 1.08 mmol/g-cells·day. The effluent sulfate ion concentration of the column in this experiment was 1.26 mg/liter even at its maximum, and the amount of oxygen consumed was calculated to be 2.1 mg/liter, even at its maximum. The saturated concentration of dissolved oxygen at 20°C was 8.5 mg/liter for the liquid of this experiment, and the influent dissolved oxygen concentration was considered to be that of

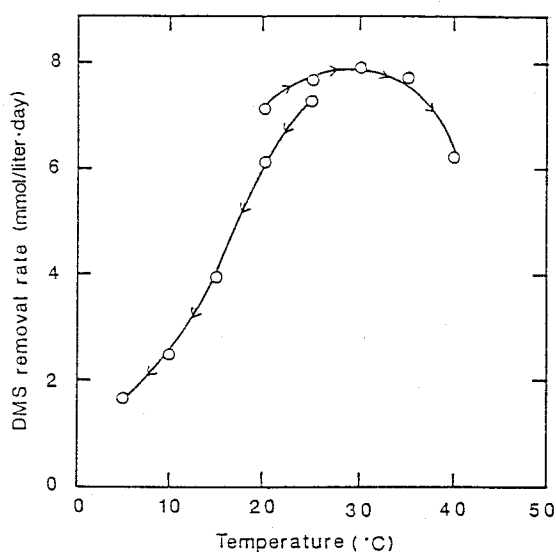


Fig. 27. Influence of Temperature on DMS Decomposition. The temperature was first raised by 5°C at 2-hr intervals from 20°C to 40°C, and then lowered by 5°C at the same intervals from 25°C to 5°C. The decomposition rates were measured at each temperature.

saturation. Hence, it is understood that the oxygen supply was not a limiting factor for the reaction in this experiment.

Influences of temperature, pH and salt concentration

The influences of temperature, pH and salt concentration were examined using the decomposition rate of dissolved DMS.

The influence of temperature is shown in Fig. 27. The optimum temperature was 30°C, at which the removal rate of DMS was 7.9 mmol/liter·day. Although the removal rate did not vary much in the temperature range of 25-30°C, it decreased sharply at temperatures below 20°C, becoming, as compared with the rate at 30°C, 1/2, 1/3, and 1/5 at 15°C, 10°C, and 5°C, respectively.

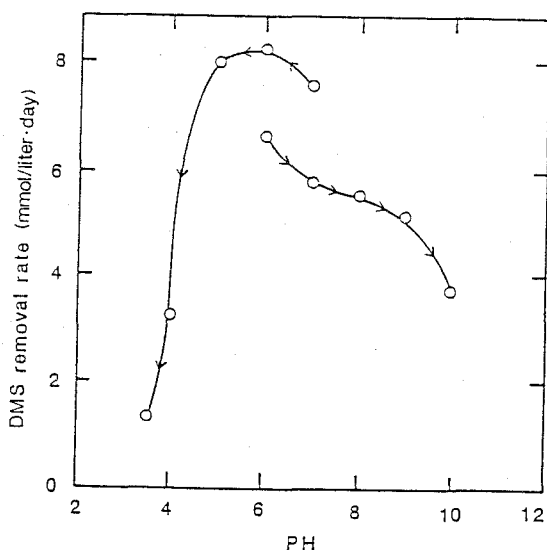


Fig. 28. Influence of pH on DMS Decomposition.

The pH was first raised by 1 at 2-hr intervals from pH 6 to pH 10, and then lowered from pH 7 to pH 3.5. The decomposition rates were measured at each pH. The adjustment of pH was carried out by the addition of HCl or NaOH to the phosphate buffer used in the experiment.

The influence of pH is shown in Fig. 28. The optimum pH was 6, at which the removal rate of DMS was 8.2 mmol/liter·day. The removal rate decreased sharply at pH values below 5, becoming 1/4 and 1/5, as compared with that for pH 6, at pH 4 and pH 3.5, respectively.

The influence of NaCl addition was then examined. The decomposition rate of DMS was 7.0 mmol/liter·day in the absence of NaCl, but decreased to 5.0 mmol/liter·day with the addition of 0.5% NaCl to the charged liquid. On addition of 1% NaCl, DMS was only slightly decomposed so that there was no increase in the effluent sulfate ion concentration.

DISCUSSION

When air containing malodorous sulfur compounds was supplied to a cylinder packed with polypropylene pellets on which T.thioparus TK-m was immobilized, a significant deodorizing effect was observed. Furusawa et al. (39) reported that the removal rate of H₂S was 0.23 mmol/liter·day when fibrous peat acclimated to H₂S was used. Author's experiment using strain TK-m produced 95% removal with loading of 17.4 mmol/liter·day, indicating that the decomposition capability was several 10-fold as high as that with fibrous peat. For MM and DMS also, this packed tower showed strong removal capability and was found to be very effective for the treatment of these malodorous substances.

The DMS removal rate per unit weight of cells immobilized on pellets was 1.08 mmol/g·day. This value is very small compared

with the rate (65 mmol/g·day) by dispersed bacteria described in Chapter 4. Since the experimental conditions such as temperature and medium composition were different, a simple comparison of these removal rates cannot be made. However, even when these factors are subtracted, the difference in the removal rates is too great, suggesting that only a portion of the immobilized bacteria contributed to the DMS decomposition. The immobilized bacteria are supposed to have formed a layer of a certain thickness, and the bacteria inside are thought to have not worked well due to the insufficient supply of substrate and oxygen. In other words, only the bacteria on the surface are supposed to have contributed to the decomposition. This is also suggested by the fact that even a great difference in cell concentration did not produce a difference in the decomposition capabilities of the packed tower apparatus. Therefore, in order to achieve a much higher deodorizing rate, it is important to select immobilizing carriers providing a wider surface.

In the decomposition experiment of dissolved DMS, the increase in DMS concentration of the liquid supplied decreased the decomposition rate. The DMS decomposition capability of strain TK-m has been shown in Chapter 3 to be inhibited by DMS itself, so it is believed there was such inhibition in this experiment also. There are no data on the DMS concentration at which inhibition appears, however, in a experiment using resting cells described in Chapter 3, the inhibition was observed at a DMS concentration of 0.1 mM (6.2 mg/liter).

Formation of sulfuric acid by the oxidation of sulfur-

containing malodorous substances lowers the pH in the packed bed. Examination of the influence of pH on the decomposition capability revealed that the capability decreased sharply at pH values below 5. The decrease in pH was suppressed by circulating liquid containing phosphate buffer in this experimental packed tower. In the absence of buffering solution, suppression of the drop in pH must be made by raising the liquid circulation cycles. However, the deodorizing capability of the apparatus was found to decrease during the period of liquid circulation. Therefore, ingenuity in the circulation method and an adequate selection of circulation cycles are important for the design and operation of the apparatus. Formation of sulfuric acid and its neutralization increase the salt concentration in the packed bed, and the increase in salt concentration was found to affect the decomposition capability. The lowering of decomposition activity caused by the increase in salt concentration should be prevented by the replacement of circulating liquid and an adequate selection of circulation cycles.

During the period of liquid circulation in the packed bed, the decomposition rate of malodorous substances decreased. This is considered to have been the result of a decrease in the interface area for gas/liquid contact, probably because the circulating liquid covered the minute irregularities on the carrier surface or gave rise to bridging between carriers. Deodorizing in the packed bed can be divided into the following three steps: ① dissolution of malodorous compounds in water, ② contact of malodorous compounds with bacteria, and ③

oxidation of malodorous compounds by bacteria. The liquid circulation in the packed bed is believed to lower the efficiency of step ① , but is essential, as mentioned above, to maintain the efficiency of step ③ .

Among the malodorous compounds in the gas liberated in night soil and sewage treatment facilities, the concentration of H_2S is high while the concentrations of MM and DMS are not that high, generally below 10 μ l/liter. When easily decomposable compounds and less decomposable compounds coexist, what usually happens is that the microorganisms utilize the easily-decomposable compounds only, leaving the less-decomposable compounds. In the case of strain TK-m, however, even when a malodorous gas containing both H_2S , relatively easily decomposable, and DMS, relatively less decomposable, was supplied, the decrease in the DMS removal rate due to the presence of H_2S was so slight that treatment of the malodorous gas containing a lot of H_2S and little DMS would pose no problem.

The influence of ammonia, which coexists with sulfur-containing compounds in the malodorous gas from night soil treatment and others, was also examined. No inhibition due to the coexistence of ammonia on the decomposability of DMS was observed.

SUMMARY

Cells of Thiobacillus thioparus TK-m were immobilized on cylindrical porous polypropylene pellets (5 mm ϕ x 5 mm) which

were packed in an acrylic cylinder of 50 mm inner diameter up to the height of 800 mm. When a sulfur-containing malodorous gas was charged to this packed tower at the superficial velocity of 0.1 m/s, maximum loading capacity (mmol/liter·day) for a malodorous gas to attain the removal rate of 95% or more was: 3.65 for DMS, 8.74 for MM, and 17.36 for H₂S. At this time, the inlet concentration (μl/liter) of the malodorous compound was: 7.44 for DMS, 17.8 for MM, and 35.4 for H₂S. For every compound, higher loading resulted in greater removal quantities. The removal rate of DMS was not overly affected by the presence of a large amount of easily decomposable H₂S.

CONCLUSION

In this thesis, the author presented microbial deodorizing processes for sulfur compounds.

In Chapter 1, degradation of DMDTP by activated sludge was described. The activated sludge acclimated to DMDTP degraded not only DMDTP but also DMTP, DEDTP, DMP and so on to P_i . Influences of pH, salt concentration and DMDTP concentration on DMDTP degradation were studied to reveal the conditions for efficient treatment of DMDTP. The wastewater from pesticide manufacturers containing dialkyl phosphates or their sulfur analogs is now efficiently treated by activated sludge processes.

In Chapter 2, DMDTP-degrading microorganisms were isolated from the activated sludge and characterized. Strain TK-1 identified as Thiobacillus thioparus grew on DMDTP at the expense of the sulfide oxidation and produced DMP and sulfate. Strain AK-2 identified as Pseudomonas sp. used DMP for growth and released P_i . DMDTP was degraded by the concerted action of these two bacteria.

In Chapter 3, degradation of DMS by a mixed culture of T.thioparus TK-1 and Pseudomonas sp. AK-2 was shown. While both these organisms persisted in stable co-culture on DMS, it was found that T.thioparus TK-1 and the derived strain TK-m grew in pure culture on DMS. During growth, all the DMS-sulfur was oxidized stoichiometrically to sulfate. DMS-carbon was probably converted to CO_2 , since the fixation of $^{14}CO_2$ was progressively diluted during growth of a culture on $^{14}CO_2$ and DMS. The

organism thus appears to exhibit a mixture, from the one substrate, of chemolithotrophic and methylotrophic energy generation supporting autotrophic growth with CO₂ fixation.

In Chapter 4, efficient removal of MM, DMS, DMDS, and H₂S from contaminated air by T.thioparus TK-m was described. More than 99.99% of DMS was removed when the load to the cells was less than 4.0 g-DMS/g-dry cell·day. MM, DMDS and H₂S were also removed as efficiently as DMS was.

In Chapter 5, in order to develop equipment capable of efficiently treating sulfur-containing malodorous gas, a deodorizing effect of an experimental packed tower apparatus containing immobilized cells of T.thioparus TK-m was examined. This apparatus was found to be very effective for the treatment.

The author constructed a pilot-scale packed tower apparatus in a sewage treatment facility and is now demonstrating the significant deodorizing effect of this apparatus. The author intends to show that the operation cost of this apparatus is much lower than those of physical or chemical processes, that the maintenance is very easy, and that the deodorizing effect is very strong and stable.

The author has no enzymological data on methyl sulfides degradation by T.thioparus TK-m. The pathway of methyl sulfides metabolism in a microorganism was first studied by De Bont et al. (6) using a DMS-degrading bacterium, Hyphomicrobium sp. S. Their data indicate that DMS is oxidized by an NADH-dependent mono-oxygenase to formaldehyde and MM, which is subsequently oxidized by a H₂O₂-producing oxidase, again resulting in the production of

formaldehyde. It is also revealed that the cell-free extracts of Hyphomicrobium sp. S contained high levels of catalase, NAD⁺-dependent formaldehyde dehydrogenase, and NAD⁺-dependent formate dehydrogenase. Recently Suylen et al. (40) purified MM oxidase, a key enzyme in the metabolism of methyl sulfides, from Hyphomicrobium sp. EG which grows well on DMS. The MM oxidase

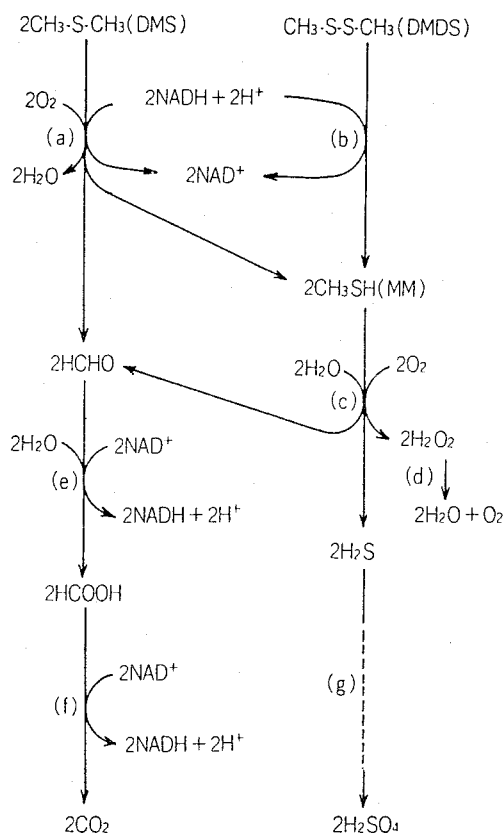


Fig. 29 Proposed Pathway of Methyl Sulfides Metabolism in Thiobacillus thioparus TK-m.

The steps are (a) NADH-dependent DMS mono-oxygenase; (b) NADH-dependent DMDS reductase; (c) MM oxidase; (d) catalase; (e) NAD⁺-dependent formaldehyde dehydrogenase; (f) NAD⁺-dependent formate dehydrogenase; (g) sulfide-oxidizing system.

catalysed the formation of stoichiometric amounts of formaldehyde, H_2S , and H_2O_2 from MM and O_2 . Smith and Kelly (41) studied the mechanism of DMDS oxidation by T.thioparus E6. Suspensions of this strain produced MM from DMDS under anaerobic conditions. MM production was stimulated by the presence of NADH as a reductant. The cell-free extracts of T.thioparus E6 contained MM oxidase, NAD^+ -dependent formaldehyde dehydrogenase, NAD^+ -dependent formate dehydrogenase, and a very high level of catalase. By analogy with the metabolism of methyl sulfides in Hyphomicrobium sp. strains S and EG and T.thioparus E6, the author presumes the pathway of methyl sulfides metabolism in T.thioparus TK-m to be as shown in Fig. 29.

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